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THE CYTOPATHOLOGY OF
VIRUS INFECTION*

Consulting Editor

ROBERT LOVE

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* This series of papers is the result of a conference on *The Cytopathology of Virus Infection* held and supported by The New York Academy of Sciences on November 7 and 8 1958

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INTRODUCTORY REMARKS

Albert S. Gordon

The New York Academy of Sciences and the Department of Biology, Graduate School of Arts and Science, New York University, New York, N. Y.

The New York Academy of Sciences has always been interested in information regarding the basic nature and actions of viruses. For this reason it has published such monographs as *Viruses as Causative Agents in Cancer* in 1952, *Virus and Rickettsial Classification and Nomenclature* in 1953 and *Viruses in Search of Disease* in 1957. Certain aspects of viral action have also been presented in the monographs *Subcellular Particles in the Neoplastic Process* and *Hodgkin's Disease* published in 1958.

Despite this interest comparatively little concentrated attention has been paid until recently to the cytopathology of virus disease. With the introduction of newer techniques such as electron microscopy, cytochemistry and fluorescent labeling, clarification of many areas of the cytopathology of virus infections has been made possible. The present monograph delineates many of these newer approaches. The information derived no doubt will further our knowledge concerning the mechanism of action of viruses on cells as well as their relation to the neoplastic process. In this connection important details of the morphologic and metabolic changes induced by a variety of viruses are emphasized.

INTRODUCTION

Robert Love
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Oscar Wilde once said that 'Nowadays to be intelligible is to be found out' At the risk of being found out I shall do my best to be intelligible Leslie Foulds of the Chester Beatty Research Institute, London, England, tells the following story about cancer research The street lamps around London end at the outskirts of the suburbs One night a policeman found a drunk crawling on his hands and knees under the last lamppost in a suburb In answer to the policeman's query, the man said that he was looking for his key Being a typical London policeman, the bobby started to look for the key After about half an hour the policeman who was sober, began to lose hope and asked if the man was quite sure that he had lost his key near the lamppost Not at all, 'replied the drunk, "I lost it out there," pointing toward the dark 'but this is the only place where there is enough light to look' When we come to consider the cytopathology of virus infection I think that we confront the same problem In effect, I have tried to bring together as many lampposts as possible in this monograph I hope that, now that they are brought together, the combined lighting effect will extend into the regions of darkness

In some ways I have been compelled to simplify the problem This monograph omits several important aspects of the virus-cell relationship There is not enough space to include the significant contributions in the fields of plant and insect viruses and of bacteriophage With the possible exception of the paper on the Friend virus, the problem of the role of viruses in the production of malignant neoplasia has not been included I think that we have problems enough, without having to consider the nature of cancer Furthermore, I feel that it may be helpful at this point to take stock of our present knowledge of what are known conventionally as virus infections, by this I mean those infections characterized by the phenomena ranging from reversible cellular proliferation and hypertrophy without necrosis to those that lead directly to the death of the cell

There are still many gaps in our understanding of the relationship between the metabolism of a virus and that of the host What is a Negri body? Antigenic material has been demonstrated in it, but nucleic acids and particles resembling virus have not yet been found What do we know about the intracellular activity of Newcastle disease virus? Particles go in and particles come out but what happens in between? We know how to prevent poliomyelitis, but we do not know exactly what the virus does, or where it is within the various cells it infects Viru like particles have been found in tumors, but nobody knows what they are doing, the viru like particle is formed after the virus has finished its work The electron microscopist is filling in many details about the morphologic evolution of the infectious particle Fundamental virological studies help to elucidate the nature of incomplete, latent, and manifest virus infection and of the genetics of viruses The formation of

techniques. It is the main purpose of this monograph to bring these varied disciplines together in the hope of achieving a better synthesis of the knowledge derived from each.

is also a useful invention. Perhaps it is time to take up arms against a sea of cloudy swelling, hydropic degeneration, and hematoxyphil bodies. Our knowledge of cytology and cytochemistry should enable us to be a little more precise. Those who say that "necrosis is just necrosis" should be reminded that "death is just death". In fact, they should be made to prove their contention. Those

proliferation followed by necrosis? What factors determine the outcome of the interaction of a virus and a cell? When we know the answers to some of these questions we shall know something of the teleological significance of changes in the cell. I can think of no better system than the study of the virus infected cell to further our understanding of cytopathology.

In conclusion, I quote some advice given by the late Welsh poet Dylan Thomas, who said: "The printed page is the place to examine the works of a poem. The platform is the place to give the poem the works." I provide the platform and leave it to other contributors to supply "the works."

A COMPARISON OF *IN VITRO* AND *IN VIVO* CHARACTERISTICS AS RELATED TO THE PATHOGENESIS OF MEASLES, VARICELLA, AND HERPES ZOSTER*

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With the recent rapid advances in virology resulting from the introduction

Salivary gland virus in tissue culture characteristically produces changes in fibroblastlike cells rather than in epithelial cells,² whereas in the human host the reverse is true. Many similar examples can be cited.

Because of discrepancies such as these, there has been little attempt to correlate cytopathic changes in tissue culture with the pathology and pathogenesis of disease. Elucidation of the causes of such differences undoubtedly will lead to a better understanding of virus diseases. Consideration of *in vitro* and *in vivo* similarities also appears to be of value, for these similarities may, first, provide evidence that a given disease is due to the agent isolated and, second, explain in part the pathology and pathogenesis of the disease in the host. In either case, the value of the feature being correlated is somewhat proportional to its degree of specificity for the agent in question. The viruses of varicella, zoster and measles each show unique cytopathic changes in tissue culture and, in each instance, there appears to be correlation between these changes and the lesions produced in the human host.

Varicella and Herpes Zoster

Most of what is known of the behavior of the agents of varicella and zoster in tissue culture stems from the work of Weller and his co-workers.^{3,7} From this work as well as other studies^{8,12} it may be assumed that a single virus is responsible for both clinical diseases. Among the evidence that such is the case is the identity of the cytopathic effect of strains of virus isolated from both entities and its unique character.

Several types of cells of human, simian, and lapine origin have been shown to be susceptible in tissue culture to this agent and to develop similar changes.⁸

Cells in suspension culture with vesicle fluid or other rounded intra-nuclear inclusions (FIGURE 1) similar to those described by Lyzzet in the skin of patients with varicella.¹¹ Occasional multinucleated cells are seen in such

* The work reported in this paper was supported in part by grants from the National Foundation, New York, N. Y.

foci in tissue culture, as well as in the skin. The infected cells show slowly progressive degeneration and may be desquamated or destroyed only after several days. The enlargement of the foci proceeds very slowly and after one week or more new or secondary foci appear and expand in turn. In cultures made up of spindle-shaped cells with an orderly or organized orientation it is noted that the shape of the degenerative foci corresponds with the prevailing orientation of the cells (FIGURE 2). Such centrifugal spread of the focus is indicative of direct cell to cell transmission and after its initial appearance, is little influenced by the presence of immune serum.⁸ The secondary foci are explained readily by desquamation of infected cells from a primary focus and reimplantation to serve as niduses for the development of these new foci. That



FIGURE 1. Tissue culture of human amnion inoculated with vesicle fluid from patient with varicella. Hematoxylin and eosin stain. $\times 467$

such is the case is suggested by the work of Weller⁹ who was able to recover infectious virus from the supernate of media from cultures showing cytopathic changes in only 1 of 8 attempts although antigen can be demonstrated in tissue culture fluid. At best then the liberation of effectively infectious virus from cells is inefficient.

The development of the intranuclear inclusions in tissue culture appears to differ somewhat from that occurring in the host in that large basophilic inclusions completely filling the nucleus are not seen. Rather inclusions appear to begin as one or more small amphophilic or eosinophilic bodies that gradually enlarge and are associated with increasingly prominent halos and chromatin margination. Such nuclear changes may occur prior to the development of changes detectable in unstained cells.

Analogies to the characteristics seen in tissue culture are readily apparent in the pathology of human disease produced by this agent.^{10, 11} In the course

of the viremia of varicella focal lesions develop in the skin and at least in fatal

as does a small group of overlying epidermal cell. In time the epidermal involvement becomes vesicular and expands (FIGURE 3). Necrosis is not as prominent in the skin lesions of chicken pox as it is in those of herpes simplex and vaccinia.

The lesions of varicella seen in almost all organs are likewise strikingly focal in character. In such lesions many different types of cells may show inclusions including the vascular endothelium. Hemorrhage is frequently present in and about these foci of necrosis as further evidence of endothelial damage. In the



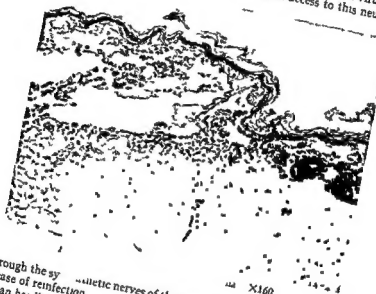
FIGURE 2. Tissue culture of human embryonic skin muscle inoculated with strain of varicella. Unstained. $\times 39$.

patient necrosis undoubtedly of viral etiology is not always accompanied by the presence of inclusions, whereas in tissue culture all the degenerating cells contain these bodies. This is particularly the case in the liver in which inclusions are demonstrated with difficulty if at all. Thus in the host the lesions somewhat resemble an embolic phenomenon with early involvement of the endothelium and local spread of the infection from cell to cell.

The comparison of the *in vitro* and *in vivo* characteristics of infection in varicella would appear complete if it could be shown that the virus is transported from the initial site of infection to the skin and other organs within one of the formed elements of the blood such as the lymphocytes. We have seen on one occasion inclusions in the thymus in cells that were thought to be lymphocytes.¹⁸ Such a carrier cell might explain in part the severity of the infections in patients with leukemia.

In keeping with the monistic theory¹⁹ of the etiology of varicella-zoster the clinical and pathological differences in the two diseases may be explained with

out invoking differences in tropism of virus strains, particularly in light of the *in vitro* characteristics of the virus. Herpes zoster typically occurs in patients who have had a previous varicella infection and who would then be expected to have a humoral immunity tending to prevent hematogenous dissemination. In shingles there is clinical and pathological evidence that spread of the virus occurs along nerves. Inclusions have been demonstrated in ganglion and satellite cells of the dorsal root ganglia corresponding to the dermatome involved, and they have also been seen¹⁸ in the sympathetic nerves (FIGURE 4). The site and mode of access to this neural pathway are not known, although it appears that herpes zoster may either represent activation of latent virus remaining from a previous varicella infection or may gain access to this neural path



X160

way through the sympathetic nerves of the respiratory or gastrointestinal tracts in the case of reinfection.

One can hardly imagine properties more ideally suited to the pathogenesis of herpes zoster than those observed *in vitro*. In addition to the inclusion bodies and the relatively slowly progressive changes leading to necrosis one might cite the focal nature of the lesions in the tissue culture the spread of the infection by cell to-cell contact and the lack of influence of antibodies in the tissue culture media on this spread. Although there must be abundant opportunities for the virus to gain access to the blood in the various lesions of herpes zoster particularly in the skin in which inclusions may be seen in the capillary endothelium secondary hematogenic involvement of the zoster lesion itself if the occur probably because of the presence of humoral antibodies. Such would not be expected to prevent the development of the zoster lesion itself if the spread is actually along cellular neural pathways.

homogeneous amphophilic material without a surrounding halo as has been illustrated by Bonenfant²² Although there are other possible mechanisms whereby such cells might originate, their striking similarity to the changes observed in tissue culture is strong evidence that the method of production of giant cells is similar *in vivo* and *in vitro*

Although epithelial giant cells in the respiratory tract have been noted in pathological descriptions²³ they did not receive much attention until their appearance in smears of nasal secretions was found to be of value as a diagnostic tool by Tompkins *et al*²⁴ We have also seen such epithelial giant cells in the mucous glands of the trachea in a typical proved case of measles dying shortly after the appearance²⁵ of the rash (FIGURE 8) Their similarity to the cytopathogenicity of measles virus in tissue culture is even more striking than

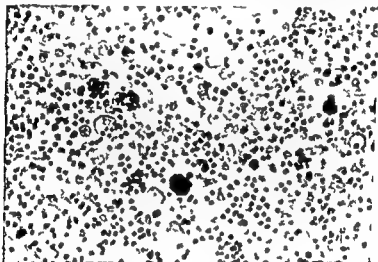


FIGURE 7 Thymus from case of measles showing development of Warthin Finkeldey giant cells X564

that of the Warthin Finkeldey cells In these multinucleated cells could be found numerous intranuclear inclusions although cytoplasmic inclusions were not evident Although there was an interstitial bronchopneumonia that was probably due to measles virus these giant cells were not seen in the lung in this case

Another lesion that may be produced by infection with the virus of measles was first described by F giant cell pneumonia
fection is more chronic

tures are quite striking and are those of a subacute or chronic interstitial bronchopneumonia with metaplasia of bronchial and bronchiolar epithelium and alveolar lining cells (FIGURE 9) From fusion of such cells large multinucleated giant cells containing intranuclear and sometimes also intracytoplasmic inclusions can be seen (FIGURES 10 11) Such cases due to measles virus can usu

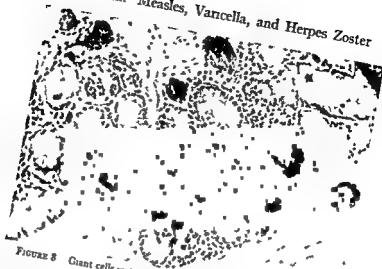


FIGURE 8 Giant cells in tracheal glands from case of measles X240

ally be distinguished from a somewhat similar pneumonia of unknown etiology²⁸ by the presence of the nuclear inclusions. That this pneumonia in children is due to infection with the virus of measles is supported by clinical association with measles infection and the morphologic similarity to the lesions of typical measles infection and the pathogenicity

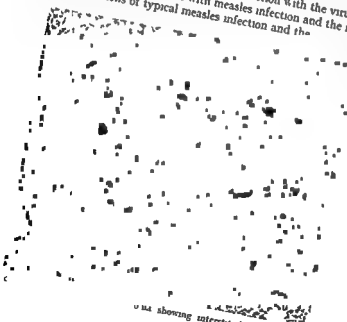


FIGURE 9
metaplasia

showing interstitial infiltrate and epithelial

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DOUBLE INFECTION OF CELLS IN CULTURE WITH MEASLES AND POLIOMYELITIS VIRUSES*

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Section of Epidemiology and Preventive Medicine Yale University School of Medicine
New Haven Conn

When a susceptible cell is exposed to two different viruses two types of reaction may ensue one virus may interfere with the penetration and/or the multiplication of the second or double infection of the cell with both viruses may occur On the basis of morphologic evidence it has been reported¹ that dual infection could be observed when one virus induced intranuclear and the other cytoplasmic inclusions but not when the inclusions induced by the two viruses had the same intracellular localization Double infection of a cell has been reported by Anderson² with fowlpox and herpes simplex with fowlpox and laryngotracheitis or with herpes simplex and rabies viruses Similar findings were reported by Syverton and Berry³ for vaccinia and either herpes simplex or B virus Double infection of a cell with vaccinia and fowlpox two viruses inducing the formation of inclusion bodies with the same intracellular localization could not be observed microscopically

In the course of studies with poliovirus in monkey kidney (MK) cultures it was observed that cultures spontaneously infected with the monkey intranuclear inclusion agent of Ruckle⁴ were apparently fully susceptible to poliovirus Poliovirus induced inclusions were seen in the nuclei that already contained inclusions induced presumably by the monkey agent Since poliovirus multiplied in cells apparently infected with the Ruckle agent as observed cytologically experiments were carried out to determine whether normal cells would support the growth of both agents at the same time The present report describes experiments on the double infection of MK cells with measles virus (which is apparently identical with the monkey intranuclear inclusion agent⁵) and with poliovirus In MK cells measles virus infection is characterized by large intranuclear and cytoplasmic inclusion bodies and by the appearance of multinucleated cells⁶ whereas poliovirus induces the formation of eosinophilic cytoplasmic masses and small intranuclear inclusion bodies⁷ These cellular alterations are quite distinct in their morphologic characteristics and changes induced by each virus can be distinguished readily

Experimental Results

After poliovirus infection of MK cells newly formed cell associated virus appears as soon as 3 hours after inoculation⁸ The growth of measles virus on the other hand is far slower and cell associated virus appears approximately 18 hours after inoculation⁹ In addition presumably because of the low infective titers of measles virus and its instability at 37° C. this agent does not

* The work reported in this paper was supported in part by a grant from The American Cancer Society New York N Y
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produce generalized cytopathic degeneration of the cells during the first growth cycle.

The experiments therefore, were designed to take the different growth rate of both viruses into account and the following experimental procedure was adopted: primary cultures of MK cells grown in Hank's saline containing 2 per cent calf serum, were inoculated with measles virus and 5 to 7 days later when the monolayers showed generalized measles induced cytopathic changes the cultures were inoculated with poliovirus. The fluids and cells from these measles infected cultures were assayed at various times after inoculation with poliovirus until the monolayer was completely degenerated. The cultures were also examined cytologically. Parallel cultures inoculated with either poliovirus or measles virus alone were harvested at the same time for comparison with the doubly infected ones. At each selected interval 6 samples from each series were harvested. 3 of the samples were frozen at -70°C for virus titra-



FIGURE 1 Cells from a monkey kidney culture inoculated with poliovirus. Early stage of cytopathic degeneration. The nucleus at the top appears normal. V nucleus with nucleolus in intranuclear inclusion. Approximately $\times 1200$.

tion the other 3 were fixed in Zenker's fluid and were stained with hematoxylin

d by measles and by

⁶ In the early stages intranuclear inclusion near darker wrinkling

of the nucleus occurs and most of the chromatin network disappears⁶ (FIGURE 1). Later a large eosinophilic paranuclear mass appears in the cytoplasm and the cell detaches from the glass and becomes rounded (FIGURE 2). Measles virus induces the formation of multinucleated giant cells with acidophilic intranuclear and cytoplasmic inclusions surrounded by a clear halo⁴ (FIGURE 3). These intranuclear inclusions differ from the ones induced by poliovirus in that they are much lighter in color, the chromatin network disappears only

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test

Cultures previously infected with measles virus are fully susceptible to poliovirus, as determined by observation of the cytopathic changes induced. When



FIGURE 2. Cells from a culture similar to that shown in FIGURE 1 but in a more advanced stage of cytopathic degeneration. *nw*, nucleus; *n*, nucleolus; *sc*, eosinophilic cytoplasmic mass. Approximately $\times 1500$.

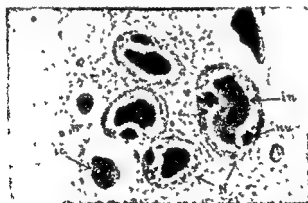


FIGURE 3. Multinucleated cell from a monkey kidney culture inoculated with measles virus. *nw*, nucleus; *n*, nucleolus; *sc*, cytoplasmic inclusion. Approximately $\times 1200$.

such doubly infected cultures are fixed and stained, changes typical of both measles and of poliovirus-induced degeneration frequently can be seen together in many mononucleated and multinucleated cells. Many cells that show wrinkled nuclei with darkly outlined membranes and eosinophilic cytoplasmic masses contain a large, light-colored intranuclear inclusion typical of measles infection (FIGURES 4 and 5). A number of these cells contain, in addition to the measles-induced intranuclear inclusion, one or several smaller and darker

intranuclear inclusion bodies similar to those seen in polio infected MK cultures, which have never been seen after infection with measles virus alone

In addition to the morphologic changes just described, it is not unusual to find in multinucleated cells that only some nuclei in a syncytium show abnormal changes or that these changes are shown to varying degrees (FIGURES 7 and 8) When such a cell contains an eosinophilic cytoplasmic mass, the

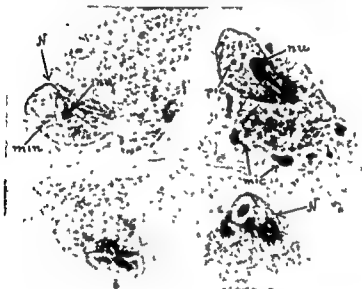


Fig. 7. Cell infected with measles virus. N = nucleus, min = eosinophilic cytoplasmic mass.

the result of superimposed cells

Virus yields from multicellular cultures Several experiments were performed using the procedure outlined above. One of these experiments will be described in detail.

Thirty six cultures were inoculated with approximately 10^4 tissue culture half dose (TCD₅₀) of measles virus, Edmonston strain. Another 36 cultures

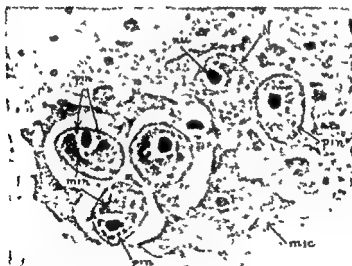
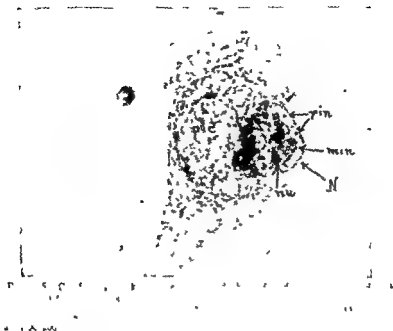


FIGURE 6
poliovirus
which the
cytoplasm
clusion. Approximately $\times 1200$



FIGURE 8. Cell from the same culture shown in FIGURE 7. This multinucleated cell contains both normal and wrinkled nuclei. Two of the wrinkled nuclei contain large measles-like inclusions. Approximately $\times 540$.

from the same lot were set aside and handled in the same manner, but were not inoculated with virus. The virus inoculated into the first set of tubes was allowed to adsorb to the cells for 4 hours at 37° C., when unadsorbed virus was removed and fresh medium was added. On the fifth day, when generalized cytopathic changes were seen and many cells had already become detached from the glass, 18 of the measles-infected cultures were inoculated with about 120 plaque forming units (PFU) of poliovirus, Mahoney strain. In the remaining 18 cultures the nutrient was changed and the tubes were reincubated. At this time 18 cultures not previously infected with measles were also inoculated with poliovirus and handled in the same manner as the doubly infected

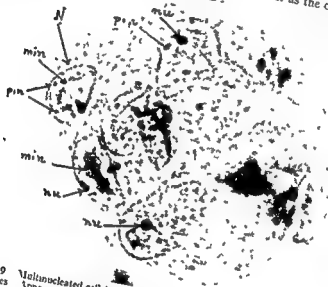


FIGURE 9
ceding figures Multinucleated cell from the same doubly infected culture shown in the pre-
approximately X1200

ones Poliovirus was allowed to adsorb on the cells for 1½ hours, the cultures were washed in saline, fresh medium was added, and the cultures were then reincubated at 37° C. Six cultures from each series 3 for cytological observation and 3 for virus titration, were harvested at 20, 48, and 72 hours after poliovirus inoculation. Titrations of measles virus were carried out in cultures of HEp-2 cells. These cells were used instead of MK because sometimes viruslike agents which appear spontaneously in MK cultures, cause cytopathic changes of the cells that cannot be distinguished from those induced by poliovirus. Titrations of poliovirus were done on MK cell fluid from doubly infected cultures, which of Dulbecco and Vogt⁴, as modified by Hsu⁵ and (asl
and poliovirus the presence of measles virus. However
poliovirus on MK cells. However
measles virus, the poliovirus pr

pathic effect of measles virus could be detected. Because of the rapid cell destruction caused by poliovirus, it was necessary to carry out titrations of measles virus from doubly infected cultures in the presence of type specific poliovirus antiserum. The samples to be assayed were mixed with equal amounts of antiserum, which neutralized at least 10^4 TCD₅₀ of poliovirus, and were kept at room temperature for one half hour before being inoculated into culture tubes. Since normal monkey serum often contains antibodies to mea-

TABLE 1
POLIOVIRUS YIELDS OF CULTURES PREVIOUSLY INFECTED WITH MEASLES VIRUS*

Hours after poliovirus inoculation	Control cultures poliovirus alone (log PFU/culture)	Doubly infected cultures (log PFU/culture)
20	5.6 5.8 5.8	5.7 6.0 6.1
48	7.7 7.8 7.8	7.7 7.7 7.8
72	7.4 7.8 7.9	Cell sheet completely destroyed at 50 hours

* Poliovirus inoculum 1.2×10^5 PFU, Type I, Mahoney

TABLE 2
MEASLES VIRUS ASSAYS FROM CULTURES BEFORE AND AFTER INFECTION WITH POLIOVIRUS*

Hours after poliovirus inoculation	Control cultures measles alone (log TCD ₅₀ /culture)	Doubly infected cultures (log TCD ₅₀ /culture)
0	3.0 3.0 3.7	— — —
20	4.5 3.7 3.7	3.5 3.0 3.0
48	4.0 3.7 4.0	3.7 2.7
72	3.7	Cell sheet completely destroyed at 50 hours

* Inoculated with 1.2×10^5 PFU of Type I, Mahoney poliovirus 5 days after measles virus infection

sles virus,¹⁰ the polio antisera used were made in rabbits and did not inhibit measles virus growth.

The poliovirus yields from the doubly infected cultures were as high as those from cultures inoculated with poliovirus alone (TABLE 1), although the number of cells present in the cultures at the time of poliovirus inoculation was lower because of the previous cytopathic effect of measles virus. In contrast, the measles virus yields were about ten times lower in the doubly infected cultures (TABLE 2). Destruction of the cell sheet by poliovirus occurred about 24 hours sooner in the doubly infected cultures than in parallel cultures infected with poliovirus alone.

The results of the virus assays support the morphologic observations and they suggest strongly that poliovirus can multiply in a cell already infected with measles virus. However cytological examination of parallel cultures infected only with measles virus and harvested at the time of poliovirus inoculation showed that about 5 per cent of the cells did not show measles induced changes as determined by morphologic criteria. The poliovirus produced by these nonmeasles infected cells could represent a large proportion of the total virus produced by the doubly infected cultures. An attempt was made to evaluate the production of poliovirus by doubly infected cells in a more precise manner by comparing the virus yields of single cells from doubly infected cultures and from cultures infected with poliovirus alone isolated in microdrops under oil according to the technique of Lwoff and Dulbecco.¹¹

Experiments on isolated single cells. This work was done with monolayer cultures of VLA cells that had been inoculated with measles virus and that showed generalized cytopathic changes with areas of cell destruction. At this time the cultures were inoculated with poliovirus calculated to give an input multiplicity of at least 5 PFU/cell. After an adsorption period of $1\frac{1}{2}$ hours the monolayers were washed and treated with disodium ethylenediamine tetraacetate (Versene) 0.02 per cent to obtain a suitable cell suspension. The cell suspension was then washed twice and resuspended in nutrient medium (Hank's saline with 0.5 per cent lactalbumin hydrolyzate and 2 per cent calf serum). Single cells were isolated in microdrops under Bayol in Petri dishes by free-hand manipulation under a dissecting microscope at a magnification of 150 \times . The microdrops then were checked carefully under the microscope to be sure that they contained only one cell. The diameter of the cells was measured by means of an ocular micrometer and the dishes were reincubated at 37°C in a humidified atmosphere containing 5 per cent CO₂ for 7 more hours. To harvest the cultures the Petri dishes were removed from the incubator and the microdrops were frozen at -20°C *in situ* in the oil. The contents of the dish were thawed just prior to titration and each microdrop was diluted in 10 or 20 ml of nutrient medium.

The cells were measured under the microscope with an ocular micrometer after isolation in microdrops and classified according to their morphologic characteristics into round cells of normal size, giant round cells, and giant flat cells. The round cells of normal size had a diameter ranging from 8 to 64 μ ; most of them measuring from 16 to 40 μ . Cells of this size were found in un inoculated cultures and in cultures infected with poliovirus as well as in measles infected cultures. The other two cell types were observed only in suspensions prepared from measles infected cultures. The giant round cells were always multinucleated and often measured several hundred microns in diameter (the few that measured less than 100 μ were not included in the data). The flat giant cells were relatively infrequent; they were multinucleated measles infected cells that had failed to round up under the action of Versene did not attach to the glass, and presumably were dead.

TABLE 3 shows the combined results of 8 such experiments performed on isolated cells infected with poliovirus alone and of 16 experiments performed on doubly infected cells. The results of the virus yields from mononucleated cells of normal size are similar in the measles and in the nonmeasles infected

cultures, the difference between them being within the range of variation found in different experiments. The methods employed have not enabled us to detect measles virus production at the single cell level, which would have made it possible to determine whether a particular single cell was doubly infected. The yields from the multinucleated cells are particularly interesting and distinctly higher: these giant cells were always multinucleated, they were never seen in cultures infected only with poliovirus, and when stained they showed the typical measles induced cytopathic changes. These doubly infected giant round cells not only produced poliovirus, but their yields of poliovirus were much higher than those obtained from small mononucleated cells. Some of these cells have produced more than 4000 PFU. Two different sets of control experiments support the view that the large amount of poliovirus associated with these cells was actually produced by them and was not merely the result of adsorption of the inoculum to the large surface of these cells. Giant cells isolated in microdrops and frozen immediately thereafter, without allowing

TABLE 3
POLIOVIRUS YIELDS FROM SINGLE CELLS

PFU per microdrop	Control drops medium % cells	Round cells of normal size		Giant round cells	
		Polio alone	Doubly infected	Polio alone	Doubly infected
0	65	100	122	—	41
1-10	3	16	43	—	11
11-100	5	37	24	—	16
101-200	0	11	3	—	4
201-1000	0	7	9	—	10
1000+	0	0	0	—	10
Total cells	73	175	201	—	92

time for virus multiplication, seldom contained poliovirus. When they did contain poliovirus, there was always less than 50 PFU/cell. Microdrops containing large multinucleated flat cells that were also found in the suspension and were presumably dead, since they failed to round up under the action of Versene and did not attach to the glass, were also studied. These cells were isolated, reincubated, and assayed for poliovirus. Thirty one giant flat cells were tested: 14 were negative, 15 yielded less than 10 PFU, and 2 yielded between 10 and 50 PFU.

All assays for measles virus produced by single cells were negative, not only in doubly infected cultures, but also in cultures infected only with measles virus. This was true even in cases when it was shown that measles virus was being produced by the monolayer before being dispersed into single cells. In 6 separate experiments, 116 mononucleated and multinucleated cells from inoculated cultures were tested for measles virus. These cells were isolated from cultures that had been infected with measles virus from 5 to 8 days previously and showed moderate to advanced cytopathic degeneration. The cells were reincubated in microdrops after isolation for periods ranging from 9 to 36 hours, but no virus could be detected. Fluid from the monolayer cultures used in

these experiments was harvested just before isolation of the same cells and contained significant amounts of measles virus which, in view of the rapid inactivation of the virus at 37°C, must have been released a short time previously.

DISCUSSION

The present data show that it is possible to infect a cell with measles and poliovirus, each of which produces alterations and inclusion bodies in the nucleus and in the cytoplasm. Previously, double infection of a cell had been demonstrated only when the inclusion bodies produced by both viruses had different intracellular localizations. The morphologic changes induced by measles and by poliovirus are quite characteristic and can be readily distinguished. Although it is quite possible that both kinds of virus particles are actually present in the same portion of the cell, our observations do not necessarily prove this. Specific virus-induced inclusion bodies are not always composed of aggregates of virus particles. In the case of measles and of poliovirus, the electron microscopic evidence available would support the view that they are not. Extensive electron microscopic observations of cells infected with these viruses have been made, and so far no virus-like particles have been found. Furthermore, the measles-induced intranuclear inclusions correspond to areas of low electron density where the chromatin network has disappeared and particles could not be seen.

These morphologic observations, which are suggestive of double infection, are supported by the results of the virus assays. Yield of the virus yield from doubly infected monolayer cultures shows that previous infection with measles virus does not interfere with poliovirus multiplication. Similarly, measles virus production continues to some time after infection with poliovirus, although the virus titers are significantly reduced. It is not possible to determine whether this reduction in titer is due to a decrease in the amount of measles virus produced by the doubly infected cells or to complete cessation of measles virus production of those cells superinfected with poliovirus.

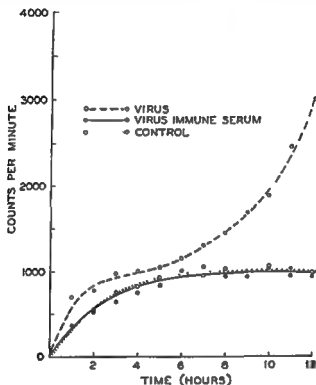
Evidence of double infection at the same cell level was obtained in the case

be explained by the large volume of these extracellular masses. However a present it is not possible to determine whether measles virus plays any role in enhancing or depressing the multiplication of poliovirus in the same cell.

ACKNOWLEDGMENTS

I thank Joseph L. Melnick and Francis L. Black for their advice and suggestions in the course of this work. I also thank Isabel Meehan for her aid in supplying the polio antisera.

This transduction phenomenon closely parallels the release of virus and the progressive loss of cytoplasmic area seen in time lapse cinemaphotography (unpublished results, W. W. Ackermann and H. Kurtz). It is probably related to the mechanism of viral release and due to specific virus action since the extent of the process in any culture is dependent upon the concentration of the viral



inoculum and can be prevented by prior incubation of the virus with viral specific immune monkey serum.

These observations tell us little of the biochemical alterations leading to this selective loss of cellular integrity, but they do determine the maximum interval after infection that can be studied most profitably. This period comprises the first seven hours when nearly all the newly formed virus is intracellular and the essential integrity of the cell is intact.

Results that are more easily subject to interpretation are obtained by following the incorporation of P^{32} , which is introduced after infection.

The object of the next experiment is to follow the incorporation into various tissue components of the labeled phosphate that was introduced at various times after infection. The essential part of the observation has already been recorded in the literature (Maassab *et al.*, 1957).

In a typical experiment, a series of cultures was exposed to a large inoculum of poliovirus. After 1 hour of incubation at 37° C, the cultures were washed free of the residual inoculum and incubated further. At various times between 1 and 7 hours after initiation of infection, each culture was exposed to P^{32} in the form of inorganic phosphate. One half hour after this addition the experiment was terminated. The cells were fractionated into cytoplasm and nuclei. Both morphologic fractions were subjected to chemical analysis. In addition, a sample of the cytoplasmic fraction was taken for virus assay. Thus the accumulated increase of nucleic acids, the rate of incorporation of P^{32} over short time intervals, and the appearance of the virus were followed.

The Nucleic Acid Composition of Normal HeLa Cells and Their Incorporation of P^{32}

When ordinary HeLa cells were fractionated into cytoplasm and nucleus and analyzed, the total ribonucleic acid (RNA) was found to be 1.5 to 2.5 times greater than the deoxyribonucleic acid (DNA). The amounts of nuclear RNA and cytoplasmic RNA were quite similar. The uptake of P^{32} into various nucleic acid fractions follows the pattern of cells from other species described previously (Marshak, 1948; Smellie *et al.*, 1953). The highest rate of incorporation is seen in the nuclear RNA followed by the cytoplasmic RNA, and DNA. The amount of P^{32} incorporated into the total RNA is 17.8 times greater than that in DNA. This phenomenon generally has been found for resting cells (Smellie *et al.*, 1953). In the experimental period studied, from 0 to 7 hours, it was found that the cell number, composition with regard to DNA and RNA, and the pattern of incorporation of the isotope did not vary significantly.

Incorporation of P^{32} into the Nucleic Acids During the Infectious Cycle

In contrast to the constant activities of the normal cell, striking changes in uptake of P^{32} occur after infection. The data in Figure 2 illustrate the rate of uptake of P^{32} during various intervals of the infectious cycle. Since the pattern of incorporation of the isotope by the uninfected controls did not vary significantly throughout the period studied, the values obtained for the incorporation of P^{32} are recorded as percentage change relative to the control. The enhanced incorporation of P^{32} by the 3 nucleic acid fractions is detectable by the first hour after infection. It is sustained in the DNA for 2 hours, after which it steadily declines but more abruptly at the fourth hour of infection. In contrast the cytoplasmic RNA increases in activity until the sixth hour, after which it also decreases. Becker *et al.* (1958) have observed also increased incorporation of P^{32} into human amnion cells following infection. Similarly, Mitroff *et al.* (1957) found an increased incorporation of P^{32} into the total nucleic acids of HeLa cells during the first 2 hours of infection with poliovirus.

Changes in the Rate of Protein and RNA Synthesis in Cells

Cultures of HeLa cells were analyzed for changes in the rate of protein and RNA synthesis. The amount of the nuclear fraction was 2.5% of the total. The amount of the cytoplasmic fraction was 12.5% of the total. The amount of the total RNA P starts by the first hour and increases to 2.5% of the total. The amount of the total RNA P starts by the first hour and increases to 2.5% of the total. Each fraction of the cytoplasm examined during the period of infection showed a 40% increase in protein. The amount of protein in protein L 7 hours represents nearly a doubling of the cytoplasmic

**RATE OF RADIOACTIVE P^{32} INCORPORATION
PER HALF-HOUR PER CELL FRACTION**

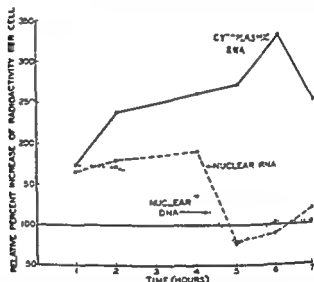


FIGURE 2 Rate of P^{32} incorporated per half hour into the 3 nucleic acid fractions. Cells were exposed to an undiluted inoculum containing a total of 1×10^6 PFU of virus for a period of 1 hour. Cultures were washed 3 times with Hank's balanced salt solution supplied with fresh medium and incubated at $3^\circ C$. The additions of P^{32} contained a total of 125 μC per culture. The amount of P^{32} incorporated is expressed as the percentage of change related to the control.

material. The first increase in viral activity in the cell was not detected until the fourth hour; extracellular virus starts to appear at about the sixth hour. It should be noted that at the seventh hour when 90 per cent of the total viral yield has been formed, only 1 per cent of the new virus is in the extracellular state. These data clearly indicate an intracellular phase in the development of poliovirus, as has been reported by other investigators (Rouman *et al.* 1958; Darnell 1958).

The increases in nucleic acids and protein described here are huge in proportion to the size of the cell. The significance of the changes can be deduced

ess of what would be incremental RNA and

protein were all virus, the yield would be 10^4 particles/cell, and the protein would correspond to 10^7 /cell. However, the yield of virus seldom exceeds 1000 plaque forming units (PFU)/HeLa cell. According to Schwerdt and Fogh (1957), the number of characteristic particles in a preparation that are countable with the electron microscope is not less than 30 PFU/cell under the best plating conditions. This would set a lower limit of 30 000 particles/HeLa cell. The increase in RNA and protein in the cytoplasm actually found at the sixth hour of infection is of a different order of magnitude than the increase in viral activity.

Second, the composition of the cytoplasmic RNA is not altered in the direction expected by the accumulation of additional RNA of the virus type. The RNA of the cytoplasm obtained after 6 hours from both infected and normal HeLa cells was hydrolyzed to a mixture of nucleotides by the method of Volkin and Carter (1951) and chromatographically separated as described by Hurlbert *et al.* (1954). The chromatographic fractionation of the hydrolyzate

TABLE 1
AMOUNT OF EACH NUCLEOTIDE IN CYTOPLASMIC RNA (cRNA) OF
NORMAL AND INFECTED HELA CELLS

Nucleotide	Amount of nucleotide $\times 10^{-4}$ per cell		
	Normal	Infected	Nucleotide*
Cytidylic acid	11.26 \pm 3.4	28.75 \pm 8.6	17.49
Adenylic acid	7.4 \pm 1.9	19.05 \pm 6.1	11.65
Guanidylic acid	10.05 \pm 3.1	38.49 \pm 8.8	22.44
Uridylic acid	8.41 \pm 1.7	21.03 \pm 7.54	12.62

* Samples for analysis were obtained from HeLa cells 6 hours after initiation of infection with poliovirus and from normal HeLa cells treated in the same manner without exposure to virus. Values recorded here are averages of data from 5 experiments.

* The difference between normal and infected cells in the amount of each nucleotide in the cRNA.

of the nucleic acids yielded the usual 4 nucleotides. The number of micro moles per cell of each nucleotide was determined. The results are presented in TABLE 1. There is approximately 150 per cent more of each nucleotide in the cytoplasmic RNA of the infected as compared to the normal cell. The nucleotide composition of the RNA isolated from the cytoplasm of normal HeLa cells is very similar to that reported for RNA isolated from other mammalian tissues in that it is rich in guanylic and cytidylic acids and poor in adenylic and uridylic acids. The RNA of the cytoplasm of infected cells was found to have a nucleotide composition that does not differ significantly from that of normal cells. However, values obtained by Schwerdt (1957) for the nucleotides of RNA from poliovirus Mahoney strain are quite different in composition from those of normal cells. The values are lower especially for cytidylic and guanylic acids. Thus while viral RNA must be synthesized in the infected cell the major portion of the newly formed RNA induced by virus infection appears not to be of the viral type but resembles more closely that of the ordinary cell cytoplasm.

Third, the distribution of the incremental materials among the various fractions does not correspond to the distribution of virus. Furthermore, in two of

the fractions the incremental protein and RNA are not present in the same proportions as in the virus

The cytoplasmic RNA is a composite of several species of RNA (Smellie, 1955) associated with various subcellular components. By differential centrifugation the subcellular elements may be fractionated and thus indicate where the incremental RNA exists in the cytoplasm of infected cells. At the sixth hour of infection the cytoplasm of normal and infected HeLa cells was fractionated by differential centrifugation, following the method of Hogeboom and Schneider (1955) into 3 fractions: Fraction I, the sediment after 6600 g for 20 min, Fraction II, the sediment after 41000 g for 1 hour, and Fraction III, the supernatant fluid above Fraction II. Each of these fractions was analyzed for RNA P, protein and virus activity, and the rate of incorporation of P^{32} into RNA.

TABLE 2
DISTRIBUTION OF PROTEIN N AND RNA P IN THE CYTOPLASMIC
COMPONENTS OF NORMAL AND INFECTED HELA CELLS

Cytoplasmic fraction	Normal			Infected			
	RNA P		mg % $\times 10^{-10}$	RNA P		mg % $\times 10^{-10}$	Virus PFU
	Counts $\times 10^4$	mg $\times 10^{-6}$		Counts $\times 10^4$	mg $\times 10^{-6}$		
I	1.45	1.95	59.74	2.73	3.21	91.52	15.5
II	1.25	4.55	54.37	2.88	13.26	73.05	313.5
III	4.45	20.38	248.19	6.54	25.51	379.87	54.5

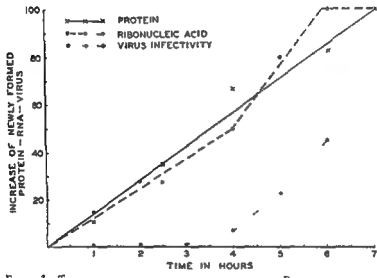
† Estimation for P^{32} incorporation by fractionation on basis of total RNA from HeLa cells 6 hours after infection.

The data are presented in TABLE 2. In the normal cell, the distribution of the cytoplasmic RNA among the 3 fractions is 7, 18, and 75 per cent, respectively. P^{32} was incorporated into the RNA of each fraction of the uninfected cell. The specific rates of incorporation of the isotope vary, Fraction III containing 75 per cent of the cytoplasmic RNA is the least active. After infection there is an increase in the rates of incorporation of the isotope in all of the fractions. Fraction II showing the greatest increase (130.4 per cent). The distribution of total RNA P follows the same pattern seen with the radioactivity data. After

fractions show an increase in protein content. The largest accumulation is in Fraction III, but the percentages in fractions I and III are the same and are greater than in Fraction II. The virus activity is found predominantly in Fraction II.

There is a characteristic value for the ratio of protein N to RNA P for each of the components of the normal and infected cell cytoplasm and for the purified virus as computed by Schwerdt (1957). A comparison of these values de-

termines whether the newly formed materials resemble the composition of the normal component with which it is associated or that of the virus or whether the materials are anomalous. Only in Fraction II does the incremental material resemble that of the virus. The incremental materials of Fraction I more closely resemble the normal components than the virus. Fraction III of the infected cells is seen to contain an increment that is relatively rich in protein as compared to the normal component. From these considerations we conclude that the accumulated protein and RNA do not constitute poliovirus and at least for RNA do represent material of the viral type (Ackermann *et al.* 1958).



following infection

Key: \times — \times — \times protein of Fraction III of cytoplasm; \circ — \circ — \circ ribonucleic acid of total cytoplasm; \bullet — \bullet — \bullet virus infectivity of total cytoplasm

Kinetics of Synthesis of RNA, Protein and Virus

The preceding sections describe the biochemical situation in the cell at the sixth hour of infection. The following experiment is concerned with the sequence of steps in time by which the situation developed. Replicate cultures of cells were analyzed at various times in the interval from the initiation of infection to the seventh hour. In order that the characteristics of development of several materials that differ in absolute amount may be compared the incremental amount of each at the seventh hour was assigned the value of 100 and the amounts at other times some proportion of 100. In this form data concerning the newly formed protein of Fraction III of the cytoplasm, the total cytoplasmic RNA and virus infectivity are plotted in Figure 3. The

synthesis of protein proceeds at a constant linear rate from the first hour until the seventh. The synthesis of RNA also begins close upon the initiation of infection and appears to be linear; the rate in the first 4 hours closely parallels that of the protein synthesis. The rate between the fourth and sixth hours proceeds at a further increased rate, and the process stops at the sixth hour. It appears that 50 per cent of the protein and RNA are formed before the appearance of any virus activity. A further 50 per cent of the virus appears after synthesis of RNA stops.

Currently, a similar study is under way. The amounts of RNA and virus were determined in HeLa cells at various times during a single sequence of infection with hemadsorption Type I virus. Some of the preliminary observations are presented in TABLE 3. From these data the following interpretations are drawn concerning the biosynthetic activities of the infected cell.

TABLE 3
PHOSPHORUS DISTRIBUTION OF HELA CELLS INFECTED WITH
HEMADSORPTION VIRUS TYPE I

Hours after infection	mg $\times 10^{-6}$ per cell*				Virus titer	
	RNA P		Total NA P of nucle		TCID ₅₀ /ml	
	C	I	C	I	Extra	Intra
2	21.59	29.9	26.90	30.90	2.5	3.5
6	—	28.33	—	32.10	2.7	3.3
12	26.13	52.45	34.2	41.50	4.3	7.0
18	—	50.22	—	49.9	5.0	7.9
24	25.99	35.2	31.0	33.0	6.8	7.7

cells were taken for virus assay

The virus induces nucleic acid synthesis in both of the major morphologic fractions of the cell (nucleus and cytoplasm). There is an increase in the cytoplasmic RNA fraction even at the second hour and a marked accumulation of RNA P between the sixth and twelfth hours. After reaching its maximum value at the twelfth hour of infection, the level of RNA P of the cytoplasm declines and the release of the virus from the cell is apparent. The nucleus on the other hand shows a sustained accumulation of nucleic acid for a period of at least 18 hours and then declines.

Associated studies of viral activity relate the appearance of new virus and nucleic acid synthesis. Viral increase parallels more closely the induced changes of the nucleic acid of the nuclear fraction. There is also an intracellular phase of viral development as found previously for poliovirus. Thus the virus seems to induce an increase of the nucleic acid of the nucleus as well as the cytoplasm of the infected cell, in contrast to poliovirus, where the incremental material is

RELATIONSHIP OF VIRUS MULTIPLICATION TO THE DEVELOPMENT OF MORPHOLOGICAL AND BIOCHEMICAL CHANGES IN THE CELL*

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A number of workers have varied histochemical and cytochemical techniques with the object of determining changes in the infected cell. The literature cited has been arbitrarily to mammalian viruses. Hyden¹ used ultraviolet microscopy to demonstrate that a number of virus infections resulted in increased deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Scott and his colleagues² have made a classic study of herpes simplex and have correlated morphological changes with the growth cycle, and have also made a study of the Feulgen reaction and observed that positive inclusions become negative with time. This was confirmed by Wolman and Behr.³ Epstein and Sautter⁴ failed to find Feulgen positive material in the inclusions of herpes simplex. Cowdry⁵ demonstrated inclusions that stained equivocally with the same technique. Blink⁶ has observed that the nuclei of human wart lesions and the intracytoplasmic inclusions of molluscum contagiosum stained more DNA than the nuclei of uninfected cells. Types 1 to 4. In the case of Feulgen microchemical analysis, the infected cells contained more DNA.

* This work was supported by a grant from the National Institutes of Health, U.S. Department of Health, Education and Welfare, during infection with the nucleus of infected cells. The growth cycle of vaccinia in the nucleus of infected cells.

clusions were noted as well as changes in nucleic acid content. The gross cytoplasmic changes and the DNA content of the infected cells were reported.

It follows that cellular changes have been observed in the infected cells.

infected HeLa cells that chemical changes by inflammation and virus infection have been observed.

* The work was supported by a grant from the National Institutes of Health, U.S. Department of Health, Education and Welfare.

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An attempt has been made to relate morphologic changes as depicted by hematoxylin and eosin (H and E) and Feulgen stains with the growth cycle of and certain chemical changes in cell nuclei.¹⁷

Three week-old Syrian hamsters are extremely susceptible to infection with EAI. Animals inoculated intraperitoneally with 1 ml each of a 1:5 dilution of freshly ground infected liver containing approximately 10⁶ LD₅₀ usually succumb with a rapidly lethal hepatitis within 18 to 24 hours. In a sequential study infected animals were compared with 2 different sets of controls: one received a comparable inoculum of normal liver the other was not injected. Following ether administration the liver and sometimes the blood were collected at intervals of 3, 6, 9, 12, and 15 hours. The livers of the remaining infected animals were collected as the animals became moribund. The tissues

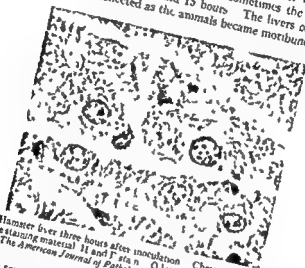


FIGURE 1 Hamster liver three hours after inoculation. Chromatin fragments are interspersed with pale staining material. H and E stain. Oil immersion. X1134. Reproduced by permission from *The American Journal of Pathology*.

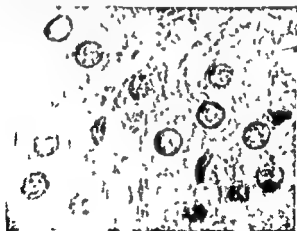
removed at the several intervals were biopsied and fixed in formalin and Zenker's fluid for H and E and Feulgen studies. The remainder was frozen and stored at -48° C for subsequent isolation of nuclei and study of the growth cycle.

Morphologic studies. Pertinent observations with H and E and Feulgen stains will be noted in FIGURES 1 to 5. At 3 hours the chromatin was distorted sometimes fragmented and interspersed with pale pink staining material. The nuclear changes were more definite at 6 hours with peripheral margination of chromatin and single to multiple inclusions were scattered about. Considerable variation in the appearance of inclusion material was observed at 9 hours. In general the changes were more advanced. Even to moderate numbers of cells resembled the 3- and 6-hour forms while the majority of nuclei were filled with basophilic material. Nucleoli usually were not recognized. In from 12 to 15 hours almost every nucleus was filled with basophilic material. Sections taken from animals sacrificed from 18 to 24 hours were very difficult to

distinguish from those sacrificed from 12 to 15 hours and often from those sacrificed from 9 hours. In scattered areas nuclear changes were infrequently more variable. The majority of cells contained full inclusions, others were



FIGURE 2 Hamster liver 6 hours after inoculation. Inclusion material is more extensive and darker staining in some cells; in others 3 hour changes predominate. H and E stain. Oil immersion. $\times 1134$. Reproduced by permission from *The American Journal of Pathology*¹⁶



adherent along certain areas of the periphery and were retracted in some degree. Scattered classic inclusions were present in some sections and absent in others. The staining of inclusions varied from basophilic to eosinophilic. Reissig and Melnick¹⁸ have reported that, after appropriate fixation and immersion in alcohol, nuclear material occurring in infected cells retracted and appeared as inclusion bodies.

The Feulgen nuclear reaction was not evident in 3 hours. Single or multiple inclusions that evidenced pale to moderate staining Feulgen reactions were noted at 6 hours. At 9 hours the relatively few Type A inclusions were usu-



FIGURE 4. Hamster liver 6 hours after inoculation. Two nuclei in lower right contain conspicuous Feulgen positive material, the larger of which appears partially retracted. Marked condensation of chromatin is conspicuous; many cells in the field are out of focus. Feulgen stain, immersion. $\times 1134$. Reproduced by permission from *The American Journal of Pathology*.¹⁶



FIGURE 5. Hamster liver 15 hours after inoculation. Field is typical of 12 and 15 hour changes. The inclusions are of dense appearance and markedly positive completely filling the nucleus in most instances. Feulgen stain. Oil immersion. $\times 1134$. Reproduced by permission from *The American Journal of Pathology*.¹⁶

ally pale to medium staining, rarely strongly positive. The most abundant were full inclusions and ranged from pale to strongly positive, the latter being more numerous. At 12 and 15 hours the overall reaction was more uniform and the majority of nuclei were filled with markedly positive Feulgen staining material. At 18 to 24 hours the size of the inclusions and the intensity of the reaction were less uniform in some areas of the slide under study. However,

it deserves emphasis that often it was impossible to distinguish between the changes seen from 9 through 24 hours. The observations are, at best, rather broad generalizations.

Growth cycle of L 41 Dilutions of blood and liver obtained from animals at the previously stated intervals were titrated in hamsters three weeks of age and the LD₅₀ determined by the classic procedure of Reed and Muench.¹⁹ The results are illustrated in TABLE 1. The data relating to the liver are representative of a number of different trials. The blood was difficult to obtain in sufficient quantity and only two trials were complete enough for comparison. It is apparent that viremia was present through the period studied and would contaminate the liver. However, enough differences emerge to permit a reasonable comparison. Under the conditions of the experiment the virus obtained from the liver rose steadily in titer from 3 to 12 hours. In either case the virus content reached a maximum at from 9 to 12 hours.

It seems valid to state that the sequential development of nuclear changes from 3 to 12 hours is concurrent with the phase of increasing virus titer between

TABLE 1
TITER OF DETECTABLE VIRUS IN LIVER AND BLOOD

Time (hours)	Blood		Liver	
	P 124	P 135	P 124	P 135
1	4 0	Not done	Not done	Not done
3	3 5	5 2	3 7	4 0
6	Not done	4 5	5 3	5 5
9	5 0	6 0	6 8	7 0
12	5 7	7 0	8 0	8 0
15	Not done	7 0	8 0	8 2

The results of hamster titrations are expressed as the negative log of the LD₅₀. P represents the passage number.

3 and 12 hours, however, the most significant changes occur between 6 and 12 hours. Also, at this time the majority of nuclei become filled with Feulgen positive material.

These results are very similar to the data obtained by Scott *et al.*⁴ It is not meant to imply that, although the morphologic changes are closely related to the growth cycle, these nuclear changes represent developmental forms of the virus. It is suggested that these changes reflect an expression of abnormal cell metabolism, a manifestation of cell injury.

Changes in nucleic acid and protein in hamsters Much of this phase of the work has been reported elsewhere.²¹ Livers from the first-mentioned study

interference.²² Protein was determined by a modification²³ of the biuret method of Robinson and Hogden.²⁴ The dry weight of nuclei is expressed in arbitrary units of 10⁻⁹ mg. The average weight of nuclei from uninoculated control

animals was 52 and did not vary significantly from the inoculated controls (animals receiving normal liver). The infected nuclei increased in weight between 9 and 15 hours to an average figure of 90. For the same time period protein and RNA changed from approximate average values of 32 to 34 and 28 to 44 $\times 10^{-9}$ mg, respectively. DNA values for control and infected animals could not be distinguished statistically, and the average was 10.7×10^{-9} mg/nucleus. The various differences between infected and control nuclei are indicated in FIGURE 6 as percentages of change from normal controls. It has been shown that the most significant morphologic changes occur between 6 and 12 hours and are concurrent with the growth cycle. The chemical

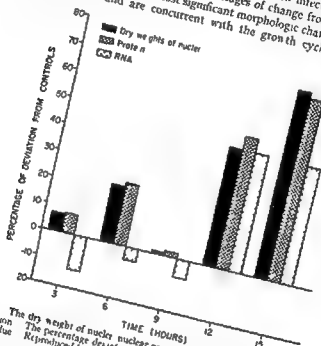


FIGURE 6 The dry weight of nuclei, nuclear protein, and RNA at various time intervals after inoculation. The percentage deviation from normal (control) is the change divided by the normal value. Reproduced by permission of *The American Journal of Pathology*.

changes lagged somewhat possibly due to the limitations of the methods. Thus effects reached a maximum between 9 and 15 hours. As yet nothing is known concerning the qualitative nature of either the protein or nucleic acid or whether they are associated. Recent data may indicate that they are combined in some way.²⁵ In our research inclusions are stained only moderately with mercuric bromophenol blue as employed by Mazza *et al*.²⁶ However following extraction of the nucleic acids with hot trichloroacetic acid the inclusions stain deeply.²⁷

Inclusion in liver lipids in 111 infected hamsters. As part of the study to investigate the chemical pathology of hepatitis in this host it seemed pertinent to study lipids. A report of a preliminary study is mentioned following IP inoculation of 20 to 25 male Syrian hamsters with 1 ml each,

of a 20 per cent suspension of infected livers, 3 animals were selected at random at 3, 6, 9, 12, and 15 hours and etherized at these times. Three uninoculated animals for each passage served as a source of control tissue. The livers were perfused with physiological saline via the portal vein, removed, forced through a 50-ml syringe into a beaker, weighed, and lyophilized. The dried pooled tissues were reweighed and extracted at room temperature with 3 changes of 20 ml of chloroform-methanol (2:1 by volume) during 24 hours. The combined extracts were chilled and evaporated to dryness. The dry residues were extracted at room temperature with 3 changes of 7 ml of petroleum ether for 30 to 45 min each. The petroleum ether extracts were pooled, evaporated with a stream of dry CO_2 , and dried under vacuum to constant weight.

Statistical analyses (student's *t* test*) indicate (TABLE 2) that significant increases occurred between 0 and 9 hours ($p < 0.05$) and between 9 and 12 hours ($p < 0.02$).

TABLE 2
LIPID CONTENT OF HAMSTER LIVERS AS PERCENTAGE OF DRY WEIGHT

Time after inoculation (hours)	Animals inoculated with suspensions of normal livers (percentages)	Animals inoculated with suspensions of infected livers (EAV)* (percentages)	Change (percentages)	<i>p</i> †
0‡	16.4	15.4		
9	10.4	18.9	3.5	<0.05
12	14.0	24.3	5.4	<0.02
15	11.0	21.0	0.3	>0.05

* Equine abortion virus

† Student's *t* test

‡ Uninoculated control

Chemical Changes in HeLa Cells Infected with EAV

It is well understood that tissue culture appears to offer a model system for biochemical and other studies of animal viruses. Fortunately, toward this end EAV was successfully adapted to the HeLa cell²⁷ which, concurrent with infection, develops conspicuous intranuclear inclusions not noticeably different from those seen in hamster liver. Cytopathogenic effects are more or less complete in 48 hours, with sloughing of the cells from the glass. Due to difficulties inherent in the problem that have not been overcome, the morphologic sequence of infection cannot be correlated with the growth cycle with any certainty. However, interesting and provocative biochemical changes which have been reported elsewhere,^{28, 29} occur in the infected cell. RNA, DNA and protein values were determined on whole cells, both infected and control values are expressed on a per cell basis. In FIGURE 7 the percentage deviation of infected cells from controls is presented. The data²⁹ subjected to statistical analysis by student's *t* test indicate that RNA was not changed, whereas DNA ($p < 0.01$) and protein ($p < 0.01$) increased significantly. Of course, no corre-

* Student's *t* test for significance. If $p \leq 0.05$ the change is considered significant.

lation with the growth cycle is implied. It is possible that, by use of cell fractionation techniques, nuclear and cytoplasmic RNA and protein may show significant changes. Such experiments are in progress.

The results of the studies with hamster hepatitis indicate interference in the biochemistry of the cell, and several of the changes during the course of infection have been measured. It is reasonable to assume that a nucleus that contains such a conspicuous mass of material that can readily be stained by a variety of methods should add some mass to the cell.

It is interesting to note that with the methods employed not all of the nuclear mass has been accounted for. This unidentified material increased significantly with infection.¹⁷ It may be inferred that the composition of the inclusion consists, at least in part, of protein and DNA, as suggested by the results of staining with mercuric bromphenol blue and the Feulgen reaction. The fact that the inclusion stains conspicuously by the Feulgen technique, while the total amount of DNA per cell is not increased, may be a contradiction of the usual

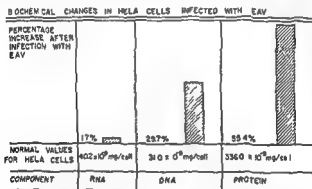


FIGURE 7. Biochemical changes in HeLa cells infected with EAV.

interpretation of the Feulgen reaction. A possibility, in the realm of speculation, is worth considering to explain the uniform Feulgen reaction of the in-

munication), who was not able to demonstrate any increase in DNA by the

value found by biochemical analysis.¹⁷

The relationship of the virus to the inclusion in this instance was purely speculative until Bracken and Norris²⁰ reported that a study of electron micrographs of EAV infected hamster liver revealed small aggregates of intranuclear virus particles that were not equivalent to the intranuclear inclusion visualized by light microscopy.

Tajima and Motohashi²¹ have demonstrated that intranuclear inclusion bodies occurring in canine hepatitis were composed of amorphous matrix mixed with numerous virus particles. The claim is made that the matrix appears to have been derived from chromatin and has been utilized for the formation of the observed virus particles.

The results of Feulgen study certainly do not permit any claims to the composition of the virus. If the virus particles from the hamster contain DNA the total amount of which is in excess of the normal amount per nucleus it is not probable that the amount of virus DNA elaborated could be detected due to the limitation of the method of analysis.

Bloch and Goldmann²² have reported a cytological and cytochemical (Feulgen microspectrophotometric) study of human viral papilloma. In the earliest stage eosinophilic nucleic acid free intranuclear inclusions are identified later becoming basophilic and Feulgen positive. These authors claim that in a later stage in the evolution of the cellular lesions all the DNA of the cell is

Sections from the same cell have been compared by the Feulgen method and electron microscopy and it has been stated that since the observed Feulgen positive crystals are composed of virus particles it is likely that the virus contains DNA.^{23, 24} In Type 5 adenovirus infection intranuclear crystals are Feulgen negative whereas surrounding virus particles are positive.^{24, 25}

In the HeLa cell DNA is significantly increased whatever the mechanism may be. Presuming that the virus replicated in the cell in question contains DNA it is unlikely that the total increase could be accounted for on this basis as an improbable number of virus particles would be required to equal the increased DNA per cell. It deserves some emphasis that adenovirus^{10, 26} and herpes virus²⁷ infections of HeLa cells are associated with conspicuous inclusions and that in each case the DNA content is increased. On the other hand infection of HeLa cells with poliovirus results in increase of cytoplasmic RNA²⁸ however Hydén found an increase in nuclear RNA and a decrease in cytoplasmic RNA. If true these data suggest that perhaps the type of cell as well as the virus may influence the nature of the cellular reaction.

The apparent constancy of DNA in nuclei derived from infected hamster liver is contrasted with the increase in HeLa cells is unexplained. It is speculative to suggest that the increase in DNA may be related to the malignant cell. In cases where DNA is increased changes in ploidy should be considered this has not been excluded in the HeLa cell infection.

It is of interest to note the results obtained with several chemical compounds. Laird¹⁶ isolated rat liver nuclei from animals previously injected with thioacetamide. In this study the DNA remained constant while RNA increased 3.5 times and protein 2.5 times. Griffin *et al.*²⁹ reported that the DNA and protein content of rat hepatic cells increased during azo dye carcinogenesis.

The nature of protein increase occurring in hamster nuclei and HeLa cells is unexplained and in either case is of such magnitude as to suggest protein synthesis. Inclusion bodies resulting from another virus infection have been shown to contain protein but in what amounts has not been stated. Bloch

and Godman²² have demonstrated that nuclear inclusion bodies contain a basic protein of the histone type. In several instances of infection, crystalline protein occurs in the nucleus and is free of DNA.^{23, 24}

Changes in Tissues Infected with Fowlpox Virus

The literature of fowlpox has been reviewed by Todd²⁵. Goodpasture²⁶ a pioneer in the study of this disease, has demonstrated that lipids accumulate and can readily be stained in lesions of the chick scalp and chorioallantoic membrane (CAM).

The morphology of the lesion has been studied with H and E stain²⁷ and by the Feulgen technique.²⁸ The inclusions were Feulgen negative until 72 hours, thereafter they remained positive as long as the study continued (12 more days). The individual elementary bodies are conspicuously positive after lipid extraction.

Nature lesions satisfactory for the purpose of this study were arbitrarily selected at 7 days and 5 days for the chick scalp and chorioallantoic membrane, respectively. Judging from the common fat stains, the amount of lipid that accumulates in the inclusion is impressive. The infected tissue seemed to be an excellent source for the study of lipids which might be expected to vary as a result of virus infection.

Lipid studies have been undertaken with other virus infected tissues. Cohn²⁹ reported decreased phospholipid phosphorus in influenza infected CAM. Cornitzer *et al*³⁰ examined papillomata from rabbits infected with Shope virus and reported a diminution in total lipid and an increase in phospholipid phosphorus.

Recently Todd *et al*³¹ have reported quantitative lipid changes in fowlpox infected tissues as indicated below. Infected scalp and chorioallantoic membrane and suitable control tissue were lyophilized to constant weight and after preliminary extraction with alcohol and Bloor's solvent were continuously extracted in a Soxhlet apparatus with diethyl ether. Combined extracts were evaporated to dryness under nitrogen and the residue extracted exhaustively with petroleum ether. The various chemical determinations were performed by standard methods. Summation of individual lipid fractions indicates that 105 per cent of the scalp and 101 per cent of CAM lipid were recovered. This is within the experimental error of the method.

The various lipid fractions from normal and infected chick skin are indicated in FIGURE 8. The total lipid contents were 23.95 ± 1.24 and 19.46 ± 0.65 per cent respectively and are calculated on the basis of tissue dry weight. The various percentages of the constituents as shown are based on the total lipid value. Statistical analysis indicates that total lipid and esterified cholesterol free and total fatty acids had changed significantly with p values < 0.05 . The decrease in total lipid may be more apparent than real as the dermis shows connective tissue proliferation and heavy infiltration by inflammatory cells thus probably increasing disproportionately the weight of the scalp tissue. The data concerning CAM lipids are shown in FIGURE 9. The total lipid of normal CAM weight was 15.98 ± 0.74 per cent and infected 23.7 per cent. The other lipid fractions are expressed as percentages.

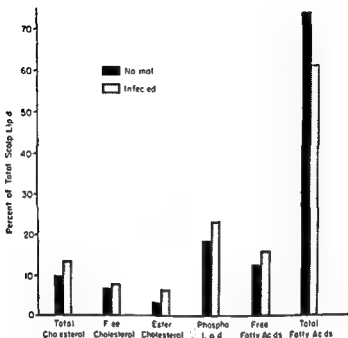
LIPID FRACTIONS FROM NORMAL AND INFECTED CHICK
SCALP

FIGURE 8 Lipid fractions from normal and infected chick scalp

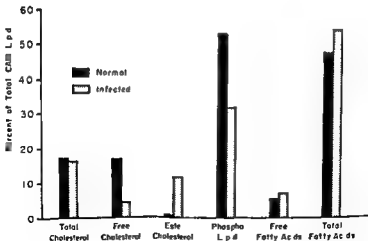
LIPID FRACTIONS FROM NORMAL AND INFECTED
CHORIOALLANTOIC MEMBRANES

FIGURE 9 Lipid fractions from normal and infected chorioallantoic membranes

lipid The p values of total lipid free and esterified cholesterol and phospholipid were <0.001

The data seem to indicate that the lipid metabolism of tissue infected with fowlpox is altered and tend to incriminate the tissues (epithelial cells) bearing the most occult lesion the inclusion body The contribution, if any, of the contiguous dermis has not been determined as yet Other sites of possible metabolic alteration such as the liver should be investigated as these may be affected despite the fact that epidermal structures are the only tissues that show specific lesions since Goodpasture¹⁶ has shown that viremia is present in the fowl

In conclusion information presented here concerning equine abortion virus and fowlpox and the work of other investigators make it evident that unexplained events occur in the virus infected cell These changes indicate significant alteration in the biochemistry of the cell Some of the components determined in the present study have diminished others have increased and in some instances probably greatly exceed values that might be accounted for solely by replication of virus particles Whatever the mechanism of these changes may be and how they are related to virus replication remains to be shown

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SOME BIOCHEMICAL EFFECTS OF INFECTION WITH FRIEND'S LEUKEMIA VIRUS

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The development of the cellular changes that a pathologist uses to characterize a disease is a step that is subsequent to and dependent upon prior alterations in the biophysical or biochemical properties of the cell. Certainly, this is no less true of virus infections than of other diseases. Indeed, in some virus infections there can be virus replication without apparent microscopic pathology.

In recent years our laboratories as well as those of others have reported some of the early biochemical alterations seen in tissue culture prior to the onset of pathology.^{1,2,3}

With regard to *in vivo* infection, the agent described by Friend⁴ as producing leukemia in mice has several attributes that commend it for a study of early effects of a virus agent. In the early stages it produces a disease restricted to the spleen so that secondary physiological alterations probably are minimized. It produces disease rapidly; pathological alterations can be seen in four or five days. The disease picture produced is quite reproducible in a very high percentage of injected mice, and the mice survive for a fairly long period of time, so that experimentation and observation can be continued. Incidentally, we refer to this disease as leukemia although we realize that some competent authorities do not agree with this designation. We were interested in trying to detect some metabolic changes that

seen cells prior to the onset of detectable pathology. It was felt that the early changes would be useful in gaining insight into the mouse nature of the disease process. There are, of course, innumerable individual metabolic processes that one might choose to examine. To attempt to cover one by one any significant part of these processes would be a formidable task. Instead, we chose to survey in one operation a broad area of metabolic activity, namely phosphorus metabolism as manifested by radioactive phosphorus uptake. It was felt that if infection produced a change in the uptake of P^{32} by the spleens, one could attack the problem in greater detail to determine if a more intimate picture of the altered biochemistry could be found.

The first part of this paper deals with our observations with whole tissue and the second part with some of the more detailed biochemistry. In order to determine if there was any alteration in phosphorus uptake as a result of infection with the Friend virus, a number of experiments were performed essentially as follows: approximately 150 white female NIH general purpose mice, approximately 18 gm in weight, were inoculated intravenously with 0.2 ml of a cell free 10 per cent extract of spleens from mice carrying the disease. The titer of the extract was about $10^{6.1}$ ID₅₀/0.2 ml. An equal number of control mice were given a similar extract from normal mouse spleen. Beginning 1 day after inoculation and at intervals thereafter, 6 infected and 6

control mice were chosen at random from the group and given $5 \mu\text{c}$ of P^{32} intraperitoneally. Three hours later these mice were sacrificed and their spleens removed, weighed wet, dried in preweighed stainless steel planchets, reweighed, and their radioactivity determined. At the same time that the spleens were removed, pieces of liver were also removed. Blood from each group of 6 mice was also taken and pooled. Some of the data from 1 experiment of this type are summarized in FIGURE 1, where the abscissa represents the number of days postinfection, and the ordinate represents the average wet weight of the 6 spleens, as well as the averaged cpm/mg dry weight. It will be seen that the spleens started increasing in size after about 4 days, but the P^{32} uptake became higher in the infected animals after only 2 days. It may

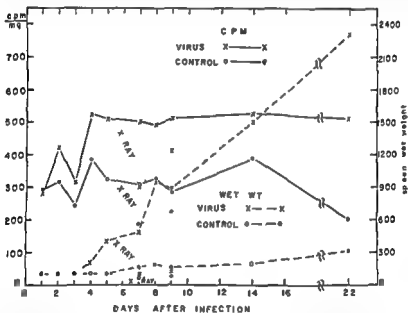


FIGURE 1. Effect of the Friend agent on uptake of P^{32} by spleen with and without γ ray treatment

be repeated here that this is not cumulative uptake of P^{32} , but rather represents just the P^{32} uptake in the 3 hours prior to sacrifice. FIGURE 2 shows another experiment where the same result can be seen. It might be remembered that at such a short interval after infection as 2 or 3 days, there can be seen little or no pathological alteration and that little virus can be recovered from the spleens.

There are a few other observations that should be mentioned. In the first place, one might ask whether the increased P^{32} uptake in the infected spleens is due to a possible alteration in spleen capsule permeability. The P^{32} is given intraperitoneally and if the spleen capsule permeability were increased as a result of infection one might anticipate the results obtained. However, when the P^{32} was given intravenously the infected tissues still took up more P^{32} per mg of tissue than did the uninfected ones.

Another point along the same line relates to a possibly higher specific radioactivity in the blood plasma of the infected animals. If such were the case, then a higher specific radioactivity in the spleens would be anticipated. Indeed, it would be anticipated for all the tissues of the infected animals. Liver and, in a few instances, brain from infected animals were examined and found to have no higher specific activity than had the controls. Direct measurement of the inorganic phosphate and the radioactivity of blood plasma and of the combined red and white blood cells revealed no consistent differences between the infected and control animals. This is illustrated in FIGURE 3. However, some evidence of a peculiar phosphorus metabolism is discernible in the wide

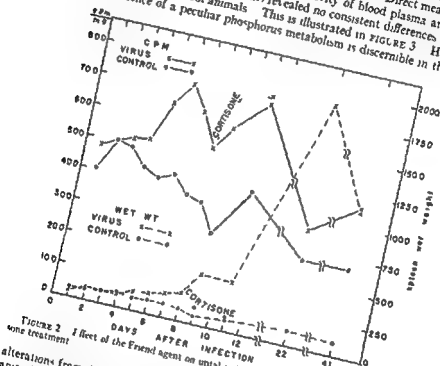


FIGURE 2 Effect of Freund's agent on uptake of ^{32}P by spleen with and without cortisone treatment

alterations from day to day in the radioactivity of the plasma of the infected animals. The controls were much more stable. Accordingly it was concluded that the most likely explanation of the stimulated ^{32}P uptake seen in the infected tissues was an effect of the virus on the tissue. Was this stimulation only a reflection of an increased growth rate? Several lines of evidence to be brought out in this paper indicate that increased growth rate certainly was not the only factor involved although it may, indeed, be a contributory one. As one observation relating to the question of growth and ^{32}P uptake, it may be seen from FIGURES 1 and 2 that the increase in ^{32}P uptake preceded by 1 to 2 days the increase in spleen size. Possibly more convincing evidence can be obtained from the work with cortisone and X-ray adjuncts to the experiment just described these 2 agents and, later, chloram

bucil were administered in the hope of obtaining at least temporary remissions as with some human leukemias and lymphomas. Cortisone at a level of 1 mg/day was given on the eighth day of infection to some of the mice used in the experiment summarized in FIGURE 2. On the following day and on the day thereafter, 6 treated control and infected mice were tested for spleen P^{32} uptake at the same time as the untreated controls and infected mice. The results of this experiment are shown in FIGURE 2.

The infected spleens stopped growing and returned to normal size. In spite

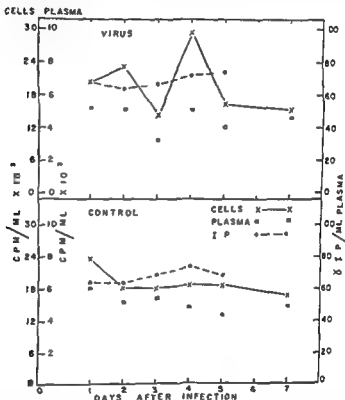


FIGURE 3 Effect of infection with the Ir end agent on P^{32} content of blood cells and blood plasma and on plasma inorganic phosphate

of this these infected treated spleens took up as much P^{32} per mg tissue as did the infected spleens from animals that received no cortisone. These results were confirmed in other experiments.

In the experiment represented in FIGURE 1, some of the mice were given 300 r of total body λ radiation on the fifth day of infection. Here as in FIGURE 2 λ ray caused the infected spleens rapidly to return to normal size instead of continuing to increase. The rate of P^{32} uptake decreased after λ ray in both the infected and noninfected animals but the λ rayed infected animals still took up more P^{32} than did the λ rayed controls. As a matter of fact as seen in FIGURE 4 the percentage increase in P^{32} uptake was just as great after either

λ ray or cortisone as it was with no therapy, even though the spleens had returned to normal size. One further observation along these lines should be mentioned. When cortisone was given beginning the day after virus inoculation, there was no sustained growth period in the infected spleens, but still the virus effect on P^{32} uptake was manifest, as seen in FIGURE 5. What can be said about the reason for the continued stimulation of P^{32} uptake in these treated spleens? If, indeed the spleens had been reduced to normal size why did they not behave like normal spleens? In the majority of cases the λ rayed spleens, although small, showed almost as large a percentage of abnormal cells as did the unirradiated ones, so that high P^{32} uptake

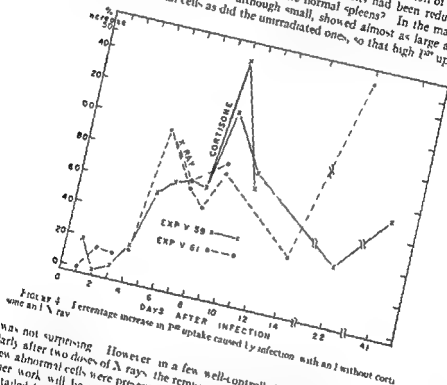


FIGURE 4 Percentage increase in P^{32} uptake caused by infection with an I without cortisone

was not surprising. However in a few well-controlled observations particularly after two doses of λ rays the remission was almost complete in that very few abnormal cells were present but a high P^{32} uptake was still found. Further work will be needed to elucidate this finding particularly along more detailed biochemical lines. The important observation from our viewpoint is that spleen enlargement is not necessary for increased P^{32} uptake. We did other experiments to test the specificity of the effect of this virus on spleen P^{32} uptake. There are quite a few reports in the literature on work done with transplanted leukemias² but the experimental conditions used were somewhat different from ours. We decided to determine whether other diseases involving either spleen enlargement or spleen pathology would show a

* One to two weeks after λ rays induced remission the infected spleens again began to grow rapidly.

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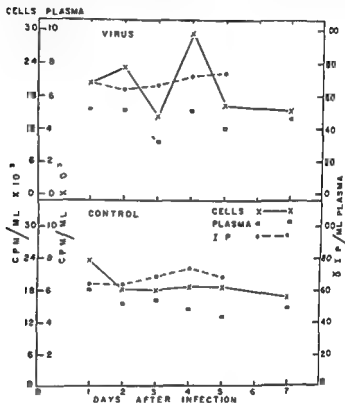


FIGURE 3 Effect of infection with the Friend agent on P^{32} content of blood cells and blood plasma and on plasma inorganic phosphate

of this these infected treated spleens took up as much P^{32} per mg of tissue as did the infected spleens from animals that received no cortisone. The results were confirmed in other experiments.

In the experiment represented in FIGURE 1, some of the mice were given 300 r of total body X radiation on the fifth day of infection. Here as in FIGURE 2 X ray caused the infected spleens rapidly to return to normal size instead of continuing to increase. The rate of P^{32} uptake decreased after X ray in both the infected and noninfected animals but the X rayed infected animal still took up more P^{32} than did the X rayed controls. As a matter of fact as seen in FIGURE 4 the percentage increase in P^{32} uptake was just as great after either

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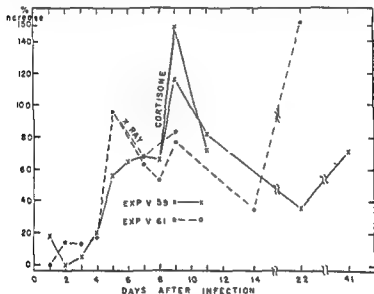


FIGURE 4 Percentage increase in P^{32} uptake caused by infection with and without cortisone and X ray.

was not surprising. However in a few well-controlled observations, particularly after two doses of X rays, the remission was almost complete in that very few abnormal cells were present, but a high P^{32} uptake was still found.⁸ Further work will be needed to elucidate this finding, particularly along more detailed biochemical lines. The important observation, from our viewpoint, is that spleen enlargement is not a necessary accompaniment of high P^{32} uptake.

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⁸ One to two weeks after X ray induced remission the infected spleens again began to grow rapidly.

similar picture to that observed with the Friend virus. The diseases studied are shown in TABLE 1 which shows that under our conditions the only additional diseases that showed increased uptake of P^{32} into the spleen were the spontaneous AKR leukemia and the lymphocytic leukemia produced by Gross virus. In other words an increase in spleen size, or pathological alterations, or even transplanted leukemia was not a sufficient condition to cause the increased P^{32} uptake that we saw with the Friend virus.

FIGURE 6 represents a photomicrograph taken of infected spleens stained by fluorescent antibody to the viral agent, by the direct procedure. Fluorescent labeled globulin from immunized rabbits was used to stain impression smears of spleens from infected mice. All sera were absorbed twice with normal liver

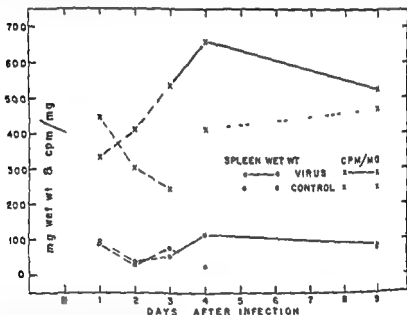


FIGURE 5 Effect of early treatment with cortisone on increased P^{32} uptake by spleens of animals infected with the Friend agent

powder and twice with normal spleen powder, and were treated with Dower 2×4 . FIGURE 6 illustrates the cytoplasmic staining seen in infected spleens. In some of the cells a nuclear and a cell membrane can be seen fluorescing. In the actual slides the nucleus was blue and the cytoplasm was yellow. Infected spleens stained with labeled globulin from rabbits immunized with normal mouse spleen and normal spleens stained with labeled antinormal spleen globulin or antiviral globulin showed no significant cytoplasmic staining and only occasional nuclear staining.

During the remainder of this paper we shall describe briefly some of the biochemical studies we have done so far with these spleens. We first studied the distribution of the injected P^{32} into several biochemical fractions. The procedure was essentially as follows. 10 to 20 mice were infected as usual by intravenous inoculation of a cell free filtrate from infected spleens. A similar num

TABLE 1
EFFECT OF VARIOUS PATHOLOGICAL CONDITIONS ON UPTAKE OF ^{51}Cr
BY SPLEENS AND THYMUS

Condition	Average wet spleen weight (mg.)		CPM/mg. dry weight		Percentage change
	Infected	Control	Infected	Control	
Lymphocytic choriomeningitis	116	127	248	219	14
Histoplasma capsulatum	503	247	285	244	13
Lymphocytic leukemia (transplanted) spleen	263	68	640	945	-35
Leukemia P 195 (Hodgkin's-like transplantable)	613	187	■	62	0
Leukemia H1-11764 G-4 (transplanted)	154	147	49	44	11
Spontaneous AKr leukemia spleen	415	75	123	67	83
thymus			174	67	160
Gross virus-induced leukemia spleen	751	57	543	322	69
thymus			396	249	60

At least 11 experimental and 6 control mice were used in 1 in most cases, 2 or 3 such groups were used. Differences of less than 20 per cent are not considered significant.

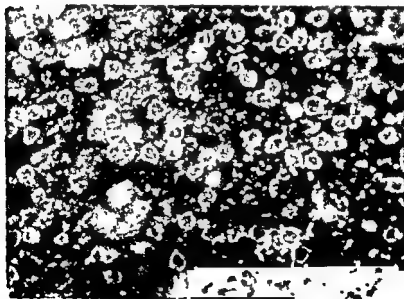


FIGURE 6 Photomicrograph of infected spleen smear stained by fluorescent antibody technique

ber of other animals received a normal spleen filtrate. After the desired period of infection the animals were given P^{32} , 3 hours later they were sacrificed and their spleens fractionated by established methods. The fractions studied and the determinations performed are indicated in FIGURE 7. From these data we get the concentration of each type of phosphorus in the tissue, the specific radioactivity of each fraction, and the concentration and specific radioactivity of the RNA and DNA. This type of observation was carried out a number of times and at different stages of infection.

For simplicity, we have summarized, from the rather large amount of data, what we feel are the most important features. TABLE 2 shows the percentages

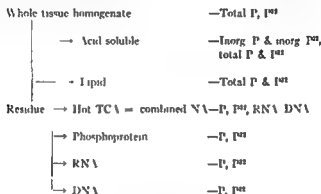


FIGURE 7 Diagram of fractionation procedure and analyses performed on each fraction

TABLE 2
PERCENTAGE INCREASE* IN VALUES FOR INFECTED SPLEENS OVER
THOSE OF CONTROL SPLEENS

	P concentration		Specific radioactivity	
	4 days	10 days	4 days	10 days
RNA	61	171	93	69
DNA	0	104	12	283

* Not absolute values

of change induced by infection in the ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP) concentrations and in their specific radioactivities for one case in which there had been relatively little

was a 61 per cent increase in RNAP concentration and a 93 per cent increase in its specific activity as compared with the values from noninfected spleens. Similarly at 10 days, when there had been considerable spleen enlargement, the infected RNAP and its specific activity were also higher than the control values. However, alterations in DNA do not appear until the spleens really had begun to enlarge. This is consistent with other literature reports in which DNA

metabolism is found to be slight in non growing tissues but becomes marked when cell division takes place.^{4, 7}

We should add that the specific activity of the lipid P fraction was also elevated in the early stages of the infection. To revert to the question raised earlier about the relationship of increased P_{32} uptake to growth, these data suggest that the effect of growth is to increase the uptake of P_{32} into the DNA whereas increase in size is not necessary for an increase in RNA concentration or specific activity. (Of course, there could also be a virus effect on DNAP, but at the moment we should not be able to detect it.

(Other evidence confirming and amplifying these observations came from experiments in which radioactive infected and control spleens were separated into nuclei mitochondria microsomes and so-called soluble fraction (or subparticulates) which, in turn were fractionated into their biochemical components namely the acid soluble lipid nucleic acid and phosphoprotein fractions. In order to simplify very complicated data for presentation here, we have prepared TABLE 3 in such a way as to show only the percentage increase

TABLE 3
PERCENTAGE INCREASE IN SPECIFIC ACTIVITY OF BIOCHEMICAL FRACTIONS OF CELLULAR COMPONENTS THREE DAYS INFECTION WITH NO SPLEEN ENLARGEMENT

Fraction	Infected	Control
Nuclei	0	0
Mitochondria	0	0
Microsomes	20	30
Subparticulates	20	40

caused by infection in the specific radioactivity of the more interesting components. In other words, we proceeded as follows. The biochemical components of the subcellular particles from infected and control spleens were separated. The amount of radioactivity and the amount of P in them was determined and the specific activity of these fractions as $\mu\text{mole mg P}$ was calculated. The percentage differences between infected and control components were calculated. These calculations are presented in TABLE 3.

These data are obtained from an infection of three-days duration. There was no increase in spleen size at this time. Examination of the summaries in the table shows that the nuclei and the mitochondria from infected tissue showed no changes in either the lipid or nucleic acid fractions as compared with controls. The microsomes and subparticulate elements from the infected spleens on the other hand showed significant increases in the specific activity of the lipid and nucleic acid fraction as compared with the control values. In the latter two fractions the microsomes and subparticulates the nucleic acid is RNA. These data show again that it is the cytoplasmic RNA that is stimulated early in infection while the DNA is not.

Current evidence suggests that the microsome and subparticulate fractions perform protein synthesis and to some extent RNA synthesis. The combination of microsome and subparticulate in a suitable *in vitro* system can

out protein synthesis,⁹ while a pH-5.2 precipitate from the subparticulates can add end groups to suitably prepared subparticulate RNA.¹⁰ It would appear from our data that, at least with respect to phosphorus, the earliest effect of the virus is to stimulate these protein- and nucleic-acid-synthesizing components. Other data not presented show that, almost simultaneously, there is an increase in activity in the acid soluble parts of the mitochondria, the energy supplying system.

A little speculation, based on these data, may prove useful. One may consider the situation in a normal cell to be such that a sort of mass action law type of equilibrium exists between nucleic acid and nucleic acid precursors, as well as between the protein for which this nucleic acid is a template and the protein precursors. When the virus infects the cell, there is good reason to think that one of the first things it does is to divest itself of its protein, if it had not already done so before entering the cell. The viral nucleic acid then may or may not break down into smaller components. We shall consider the smaller component concept first. If we were to hypothesize that these smaller components attach themselves to the subparticulate RNA of the cell, possibly through the mediation of the pH-5 enzyme just mentioned, then the equilibrium situation is destroyed. There are new templates for RNA and thence for protein, and new demands are set up but these are now demands for virus RNA and virus protein. The cell knows only that it needs to make more RNA.

into the picture. It is unusual but it does have the charm of a

In conclusion, we repeat our model for study in the virus infection. I am of the opinion that a chemotherapy tool for the protein and

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We thank Ruth Kirschstein for the pathological evaluations provided and Victor Haas, Hubert Hasenclever Lloyd Law, and Ludwig Gross¹¹ for supplying many of the animals used

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CYTOPATHOGENIC EFFECT OF TWO NEWLY RECOGNIZED MYXOVIRUS STRAINS: MECHANISM OF SYNCYTIAL FORMATION

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The recent isolation of numerous newly recognized myxoviruses^{1,2} demonstrates the heterogeneous composition of this group, since they were found in patients ill with diseases such as mumps, influenza, or croup. Myxoviruses may produce in different tissue culture systems (monkey kidney cells, Kb cells) different manifestations that may be described schematically as follows: (1) little or no cytopathogenic effect but the proliferation of the virus may be detected by the hemadsorption technique, as described by Vogel and Shelokov⁴ and (2) definite cytopathogenic effect (CPE). At present two different types of CPE may be recognized: the development of virus induced giant cells and the development of intracytoplasmic inclusion bodies. These two types of lesions may be found either associated or alone; this property depends on the virus strain examined and also on the tissue culture system chosen for the propagation of the virus.

Recent studies in this laboratory were focused on the mechanism of cytological alteration produced by myxoviruses. Two recently isolated myxoviruses were chosen for their capacity to induce typical or rapidly extending cytological lesions: the Greer strain of CA virus isolated and described by Chanock, and the Mills strain of the HA 1 viruses isolated by Rowe and Huebner and recently identified by Chanock.³ These were used as models for this study. For comparison a similar virus, apparently belonging to the HA 1 virus group and isolated in March 1958 in this laboratory, was also used.³ The study was carried out with microcinematographical and cytochemical methods.

MATERIAL AND METHODS

Monkey (*cynocephalus*) kidney cells were grown on coverslip preparations in Leighton tubes with a medium containing 45 per cent Hanks' solution 50 per cent modified 199 medium, and 5 per cent inactivated horse serum. The same medium was used for maintenance, but without serum.

Kb cells were grown in casein hydrolyzate medium⁵ with 10 per cent horse serum in the maintenance medium horse serum was replaced by 2 per cent chicken serum.

Mills strain of the HA 1 virus was propagated in Kb cells (2×10^6 ml containing 100 CPD₅₀) and checked under the microscope every 6 hours to detect early cytopathogenic effect. When early lesions appeared, the coverslip was mounted in a plastic chamber and sealed with paraffin. Progression of the lesions was observed at 37° C with a Zeiss microscope and pictures were taken.

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at a speed of 6 frames/min. Uninoculated control Ab cells were observed under the same conditions. The observation period averaged 18 hours.

CYTOCHEMICAL TECHNIQUES

Nucleic Acids

Euglen staining After fixation with Helly fixative and elimination of Hg with Lugol's iodine solution, the slides were hydrolyzed for 8 min with HCl (1%) at 60° C, and then washed in distilled water at 4° C to stop the reaction. The slides were immersed in Schiff's reagent for 90 min and rinsed 3 times in a solution of sodium bisulfite (10 per cent), 10 ml HCL (1%), 10 ml, and distilled water 200 ml.

Methyl green-pyronine and ribonuclease test Fixation in acetone for 2 hours, at 4° C. The coverslip was mounted on 2 matchsticks on a wet filter paper, and the whole was placed in a Petri dish. The preparations were immersed in a ribonuclease solution 1:5000 in phosphate buffer at pH 7 at 55° C for 1 hour. Control coverslip preparations were placed in the same conditions and covered with a phosphate buffer solution. Both coverslips were then stained with a methyl green pyronine stain for 30 min and washed quickly with ethanolic acetone, 50:50, followed by pure ethanol.

Toluidine blue and ribonuclease test The same procedure was used, except that the coverslips were stained with toluidine blue 0.1 per cent in an acetic buffer (1/15) at pH 3 and at pH 4.

Proteins

Vanillin reaction (Roman procedure) Preparations (Lyon) were fixed in 10 per cent formalin fixative and placed in boiling 0.4 per cent vanillin solution for 1 min. Cells were observed after mounting in glycerol.

SH groups Coverslips were fixed in 10 per cent formalin containing 5 per cent trichloroacetic acid (TCA) for 1 hour or more and stained without any fixation. Two tests were performed simultaneously in cells with blocked SH functions: blockage being produced by using HgCl₂ for 1 hour and in cells with free SH functions, which were subjected to 3 baths: 5 min each of K ferricyanide (0.1 per cent), 2% ml and Fe₂(SO₄)₃ (1 per cent) 75 ml in the dark. After washing in distilled water preparations were mounted in Canada balsam.

Fats

After fixation with 10 per cent formalin cells were washed with 70 per cent ethanol and stained for 15 min with a saturated solution of Sudan black B in 70 per cent ethanol.

Polysaccharides

Preparations were stained by the p-aminosalicylic acid (PAS) method after fixation in Helly solution or 90 per cent ethanol and 10 per cent formalin. Cells were oxidized for 5 min with 5 per cent periodic acid as described by McManus. The presence of glycogen was eliminated by the amylose test.

Incorporation of 35S Methionine

For the study of incorporation of ^{35}S methionine $0.25\text{ }\mu\text{C}$ of *dl* ^{35}S methionine/2 ml of tissue culture medium was added. After 2 hours of incubation the cells were fixed with 10 per cent formalin. Autoradiography was performed using an Ilford G5 emulsion. After 24 hours exposure in the dark at 4°C the plate was developed with the Kodak D 19b stock solution and fixed. Staining of the nuclei was performed with Mayer's hematoxylin.

RESULTS

The production of multinucleated cells : Altogether six series of experiments were performed. In all of the six tissue culture passages in this laboratory the Mills strain of the HA 1 virus group induced the formation of giant cells. No intracytoplasmic or intranuclear inclusions were found in H and E stained preparations. The Feulgen reaction did not demonstrate any abnormal concentration of deoxyribonucleic acid (DNA) in the nucleus at the early stages of the syncytial formation. In the late stages some of the nuclei in the clumped syncytial areas showed the presence of some pyknotic nuclei. Neither did the methyl green pyronine stain show after the ribonuclease test any abnormal concentration of ribonucleic acid (RNA) when compared with control preparations.

In unstained preparations the development of giant cells was observed by microscopic and cinematographic techniques. One hundred DCP₄₅ of virus was used in these experiments. The giant cells appeared in any area of the cell sheet (FIGURES 1 and 1a). During the early stages the number of the nuclei contained in each of the syncytial areas was limited to a few. At the time of extension of the giant cells more and more nuclei were gradually enclosed in the syncytium (FIGURES 2 and 2a) and in more advanced stages several hundred could be numbered. Thus in 48 hours about 50 per cent of the cell area showed typical alterations. After 48 hours some of the syncytia started to

as is to be noted that no mitotic figures were observed on the syncytial areas although they were frequently seen in the control tubes and in the noninvaded region of the test tubes. Similar lesions were observed with the FA 102 strain isolated in this laboratory a strain of virus that probably also belongs to the HA 1 group.

The number of the syncytial areas was proportional to the titer of the virus present in the culture under experiment and was remarkably constant from one experiment to the other. It has been postulated therefore that each of the syncytial areas is produced by one infectious unit.

As shown by the time lapse motion pictures the mechanism of giant cell formation is not atypical mitosis as sometimes suggested but a cell agglutination or conglomeration that may be described as cytophagia. A similar phenomenon has already been observed and described with some adenovirus strains especially those with slow growth.¹⁰

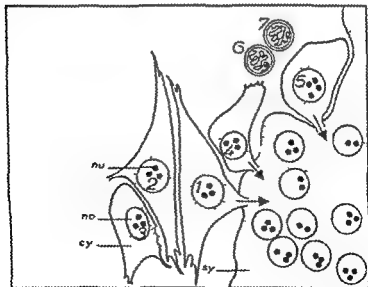
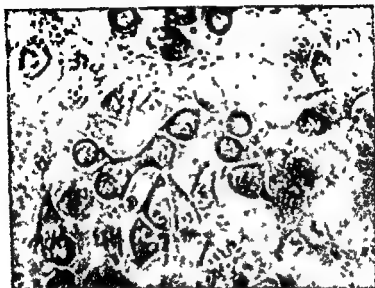
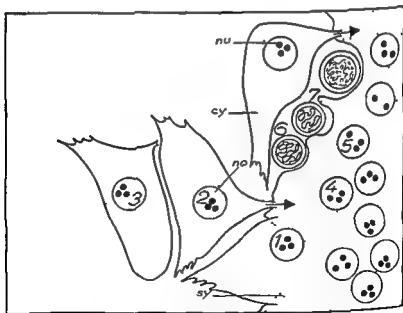


Fig. 1 and 10. The same from the left showing the nucleocapsid of the virus. The arrows 1, 2, 4 and 5 to be cytoplasmic. The arrows 3 and 6 to be cytoplasmic. The arrows 7 and 8 to be cytoplasmic. The arrows 9 and 10 to be cytoplasmic.



FIGURES 2 and 2a. Frame from microfilm showing KB cells infected with M115 strain of HA1 viruses. cells 1-4 and 5 are already included in the syncytium. mitotic cells 6 and 7 are resistant to the cytopathic effect after falling of the syncytium these will remain attached to the glass.

There is an increase in cytoplasmic volume at one extremity of the virus-parasitized cell. Contact with a neighboring cell produces attraction of the latter. When the two cytoplasmic membranes are in contact, a narrow communication is established between the two cells. The cytoplasmic content of the invaded cell is attracted into the apparently virus-parasitized cell. Shortly afterward the nucleus of the cell is incorporated without any obvious morphologic damage to it. During the progress of the giant cell formation, some of the cells resist cytophagia. In some instances these cells show mitotic figures. In late stages, when the syncytium detaches itself from the glass these resistant cells still remain attached to it.

The cells included in the syncytia do not show any sign of cellular death. When stained with 0.04 per cent erythrosin no penetration of this stain in the syncytial areas was observed after 1 hour. After 48 hours, the pH of the test tubes seemed to be slightly more acid than that of the control tubes. As shown by the autoradiographic experiment, the ^{35}S methionine apparently was incorporated in greater amount in the syncytial areas than in the noninvaded areas and the control cells.

CYTOCHEMICAL STUDY OF CYTOTOXIC INCLUSION BODIES

Nine series of experiments were performed and thus over 160 stained preparations were examined.

(1) Produced by the group associated (C 1) virus has been described by (1) Hancock in unstained preparations. In monkey kidney monolayer tissue culture this virus produces "syncytial areas with loss of the cell boundaries followed by the breaking away from the surrounding cell sheet." Then small vacuoles make their appearance and the cell loses its structure, producing a picture best described as sponglike.

In hematoxylin and eosin stained preparations from the first passage, we observed syncytia containing eosinophilic intracytoplasmic inclusion bodies that were not present in the uninoculated tissue cultures. These lesions were regularly present in the nine subsequent passages performed in this laboratory (Figures 3, 4, and 5).

In the early stages of the progression of the lesions inclusion bodies were of a small size, rounded and clearly limited. In more advanced stages, several inclusions were observed in the same cell and, in some instances, the fusion of these inclusions resulted in a larger inclusion always sharply limited surrounding by an unstained area of irregular shape and frequently situated close to the nucleus. When high magnification with immersion oil is used, these eosinophilic inclusions showed a granular structure. No change was observed in the staining affinity of the inclusions in late stages when H & E staining was used. Eosinophilic inclusions were also observed in the cytoplasm of uninoculated control cells, but they were not transmissible, they were much smaller and far less numerous than the inclusions observed in the infected cultures.

While this study was in progress Brandt¹⁰ reported similar observations with Newcastle virus and with some strains of mumps virus. Both of these viruses belong to the myxovirus group. The Swiss cheese-like appearance described

the small eosinophilic inclusions sometimes present in the control tubes were not clearly demonstrated in this series.

The ninhydrin reaction was weakly positive, but a striking fact was the strong positivity of the SH groups as demonstrated by the Chevrement-Frederic reaction. The significance of this finding will be discussed later.

The PAS test showed the presence of polysaccharides or lipids in the inclusions in a very irregular manner. The PAS reaction was unaffected by amylase digestion and by toluene extraction. Sudan black B staining showed only a very small amount of lipids in the inclusions.

DISCUSSION

Insofar as we know, the first observation of viral cytophagis in tissue culture was described in the P 134-4 strain of adenoviruses in HeLa cells by the simple study of stained preparations.¹¹ In the case of adenoviruses (chiefly slowly growing strains), agglutination by a virus parasitized cell of another healthy or

did all often not certain destruction of the nucleus of the invader,

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issue culture by physical and chemical agents such as Koch bacillus extracts,¹² but in such cases none of them is transmissible by filtrates. Intracytoplasmic inclusions observed in our series are very similar to those described with measles. More information is still needed to evaluate the complete significance of these findings, but it seems worthwhile to consider the type of cell injury as a definite part in the characterization and description of any virus.

SUMMARY

Recent reports on the isolation of newly recognized myxoviruses have shown the heterogeneous composition of this group. Myxoviruses may produce such unrelated clinical manifestations as mumps and respiratory infections. In different tissue culture systems (cytorepithelial, monkey kidney or K₈₃ cells) myxoviruses may show either (1) proliferation without cytopathogenic effect but "enveloping" (2) multinucleated giant cells, or (3) intracytoplasmic inclusions with or without giant cells. A study is made on the basis of cinematographic and cytochemical observations of the mechanism of C_{PL} produced in the latter two cases. Two newly recognized myxoviruses, H₁ and C₁, have been studied as models. The H₁ virus induces the production of multinucleated giant cells but no inclusion bodies; this giant cell formation is the result of cytophagia as demonstrated by a cinematographic study. The syncytium is produced by progressive extension of this phenomenon from one cell to the other. Each viral area can be considered as resulting from one infectious unit. H₁ virus produces giant cells and intracytoplasmic inclusion bodies in monkey kidney tissue culture. These are eosinophilic when stained by hematoxylin and eosin; they are irregularly shaped granular in structure morphologically related and closely similar to the intracytoplasmic inclusions produced by measles. The chemical composition of these inclusions was investigated by histochemical methods. There is good evidence that they contain a high concentration of RNA and DNA groups. The question arises as to whether these virological changes are to be considered as specific characteristics of myxoviruses.

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We express our gratitude to K. Lave for help and criticism, to W. P. Rowe and K. Huclner for making the Mills strain of H₁ virus available and to Robert Chanock for the C₁ strain of myxovirus. We also thank Pierre Mandl for the cinematographic division of the Institut Pasteur for his collaboration in making the motion pictures and microphotographs used to illustrate this paper.

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QUANTITATIVE CYTOCHEMICAL INVESTIGATIONS ON THE EFFECT OF VIRUS ON CELLS*

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The effects of viruses on cells have been studied by a great variety of methods, and many contributions to the host cell virus relationship have come from immunology, biochemistry, tissue culture, and other disciplines. One of the problems in which we have been interested for a considerable time concerns the behavior of the nucleoproteins, especially that of the deoxyribonucleic acid (DNA) in cells after viral infection. A fruitful pathway for the study of this particular problem appeared to be the application of quantitative cytochemical techniques such as microspectrophotometry and interference microscopy.^{1,2} The development of this new type of microscopy is based largely on the pioneering work of Caspersen,³ who demonstrated that by extending the optical properties of the microscope into the analytical sphere, the microscope can be used simultaneously as an instrument for both morphologic and chemical analysis of cells. Since the chemical analysis can be done in microscopic preparations *in situ* that is without destroying either the architecture of cells or their relationship within a tissue a direct comparison of cell morphology with chemical composition can be made directly under the microscope. Although methodological details cannot be discussed here, it should be mentioned that the basic principle of microspectrophotometry especially when applied to the determination of DNA in single cells, is actually very simple. The Fuigen reaction that leads to a specific staining of the DNA is carried out on a wave length characteristic for the DNA dye complex is used as a basis for the qualitative and quantitative analysis of the stained cell structure. Thus microspectrophotometry closely resembles analytical chemistry and a microspectrophotometer such as the one that we use in our own laboratories for DNA determination and shown in Figure 1 converts actually only of a light source L, a monochromator or filters F to isolate the desired wave length the microscope M, the photometerhead with phototube P, the power supply P and galvanometer G, these permit the measurement of the amount of light absorbed by a single cell structure. Light coming only from the cell structure in which the DNA is to be determined is permitted to reach the phototube, the remainder of the light transmitted is obliterated by a diaphragm D.

The unique features of this method lie not only in the possibility that a DNA quantity as small as 3×10^{-9} mg can be determined in a well preserved single cell structure,⁴ but that it is also possible to detect differences in the DNA content from cell to cell that may occur when cells undergo certain changes (be they cause normal or abnormal) such as meiosis, mitosis, degeneration or as will be discussed in this paper when cells are infected with virus.

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At present the conventional biochemical methods, in spite of their great contributions to the nucleic acid field do not permit the study of quantitative DNA changes at the single cell level because their analyses must be done on relatively large cell populations where individual cell morphology is destroyed and therefore of necessity the biochemical technique can yield only average values.⁸⁻¹⁰

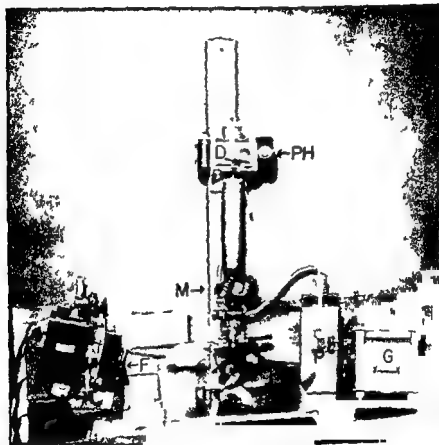


FIGURE 1. Microspectrophotometer

Turning now to the quantitative cytochemical investigations on the effect of viruses on cells it should be pointed out that this paper will deal only with the behavior of the intranuclear DNA in human cells infected with viruses known to contain DNA.

In view of the importance of DNA as an essential chemical constituent of cells, its great significance for cell life, its close relationship to the genes, and its quantitative stability in normal cells, the study of DNA in cells after they have become infected with a virus which itself contains DNA is obviously of great interest when attempting to elucidate virus-host cell relationship. Since

the question arose whether the response of the intracellular DNA to the virus DNA is a more or less characteristic one, different types of cells infected with different DNA-containing viruses were studied and two main lines of approach were followed. The first line concerns the DNA behavior of cells *in situ* of tissues from human hosts with virus diseases, the second line concerns the DNA behavior of cells in tissue cultures infected with virus.

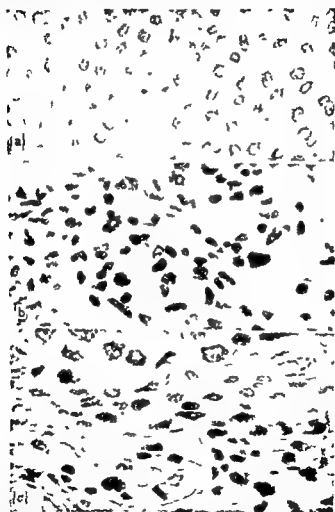


FIGURE 2. (a) Cells of normal human skin (malpighian layer). Feulgen stain. Approximately $\times 400$. (b) Cells of human skin infected with verruca vulgaris. Feulgen stain. Approximately $\times 400$. (c) Cells of human skin infected with verruca vulgaris. Feulgen stain. Approximately $\times 400$.

One of the well known virus diseases of the human skin is the common wart *verruca vulgaris*. The characteristic appearance of such infected skin cell under the microscope is seen in FIGURE 2b and c. In contrast to the uninvolved epithelial cells (FIGURE 2a), the infected skin cells (FIGURE 2b and c) show large nuclei, filled with bizarrely shaped, often grapelike masses of DNA-containing material or crystal like structures. When nuclear size and DNA content are determined in individual cells of normal skin and compared with those

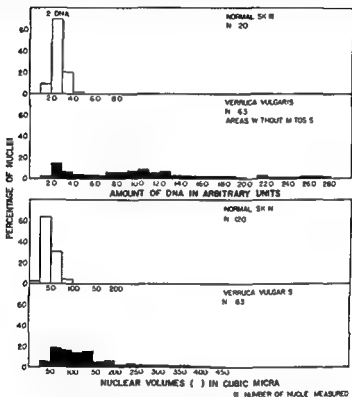


FIGURE 3 Comparison between amounts of DNA (microspectrophotometry) and nuclear sizes of individual cells from normal skin and skin infected with verruca vulgaris

infected with verruca vulgaris data are obtained of which a characteristic example is shown in FIGURE 3. It is evident that the nuclear sizes and the DNA values (as obtained by Feulgen microspectrophotometry) of the normal skin cells show a relatively narrow range that is relatively little variation from cell to cell. The mean DNA value corresponds to the characteristic basophilic

the cells of the skin infected with verruca vulgaris exhibit not only larger and variable nuclear sizes but also strikingly higher DNA values with considerable variability of the DNA content from cell to cell. It thus appears that infection

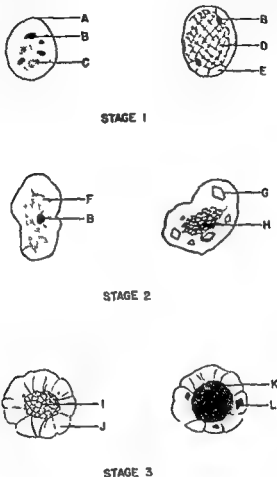
with verruca vulgaris interferes with the stability of the DNA content of the normal skin cells and elicits production of abnormally high amounts of DNA within the nuclei. Since these increased quantities of DNA were found in areas of skin without mitosis, the high DNA amounts cannot be explained on the basis of an abnormally rapid cell division, as Leuchtenberger *et al*¹¹ have reported to be the case in human tumors. It is also highly improbable that polyploidy or polypeny may be responsible for these high DNA values because, instead of well-defined multiple groups of 2DNA, 4DNA and 8DNA such as,

and c)

In order to determine whether this abnormal cytological and cytochemical DNA pattern is specific only for skin cells infected with verruca vulgaris in the human host, human cells of different tissues infected with different DNA-containing viruses, namely adenoviruses, were studied in tissue cultures. Tissue cultures are very favorable systems for such investigations, since the sequence of events in cells can be assessed from a cytological and cytochemical point of view at various known time intervals after the viral infection.

In earlier studies by Boyer *et al*¹² HeLa cell tissue cultures were infected with adenoviruses (Types 1, 3 and 4). The characteristic stages of nuclear changes in HeLa cells infected with Type 3 or 4 are presented schematically in FIGURE 4. The increase in nuclear size, the formation of bizarrely shaped masses of DNA and the occurrence of DNA containing crystal-like structures are all features similar to those in verruca vulgaris. Quantitative DNA determinations by Feulgen microspectrophotometry also gave higher DNA values for the HeLa cells infected with adenovirus than for the uninfected HeLa cells. However, HeLa cells are not a very suitable cell type for comparative quantitative DNA studies. This is due to the fact that the DNA values in the interphase nuclei of the noninfected HeLa cell control culture show relatively high and variable amounts (their basic value being 4DNA) from cell to cell, which of course is not surprising since we deal with cancer cells undergoing rapid, often abnormal divisions. Therefore, it seemed desirable to use another human cell type, preferably a normal cell with a basic diploid DNA content and with a relatively slow mitotic process. Amniotic tissue cultures, which we prepared according to the technique of Dunnehacke and Zitter¹³ were very favorable material for such studies. The cytological features of uninfected amniotic

was infected with the adenovirus. The characteristic DNA values of the interphase nuclei of different periods after infection are presented in FIGURE 6. In order to establish a base line, the DNA values were determined first in sections of the amnion fixed immediately after it had been obtained from the



delivery and before it was trypsinized and used for tissue culture. It is evident that the amnion contains cells with a basic 2DNA content, but that there are also a few cells with higher DNA values, which are between two and four times the basic values. A picture similar to that of the amnion sections is obtained when the DNA content was determined in the first generation tissue cultures of the same amnion, here again cells were found with DNA contents between



FIGURE 4. (a) Amniotic cells of control cultures (first generation in amnion No. 5) Feulgen stain. Approximately $\times 400$. (b) Amniotic cells 72 hours after infection with adenovirus Type 7. Feulgen stain. Approximately $\times 400$. (c) Amniotic cells 96 hours after infection with adenovirus Type 7. Feulgen stain. Approximately $\times 400$.

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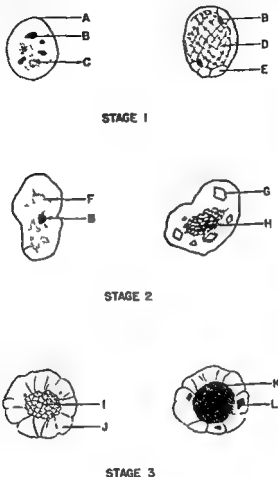


FIGURE 6. Amnion sections infected with adenovirus. (C) control; (G) granular; (I) interphase; (K) metaphase.

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FIGURE 5 (a) Amniotic cells of control cultures (first generation amnion No. 5) Feulgen stain Approximately X400 (b) Amniotic cells 72 hours after infection with adenovirus Type 7 Feulgen stain Approximately X400 (c) Amniotic cells 66 hours after infection with adenovirus Type 7 Feulgen stain Approximately X400

2DNA, 4DNA, and 8DNA. Whether the DNA values that are higher than 4DNA are due to synthesis of the diploid DNA cells or whether they are due to the fact that the amnion contains a few tetraploid cells that synthesize DNA cannot be decided at present, since mitotic figures were so scant that chromosomal counts could not be made. The possibility that the amnion does contain polyploid or polytene cells, that is, nuclei with 2DNA and 4DNA content must be considered seriously, since we know that normal tissues

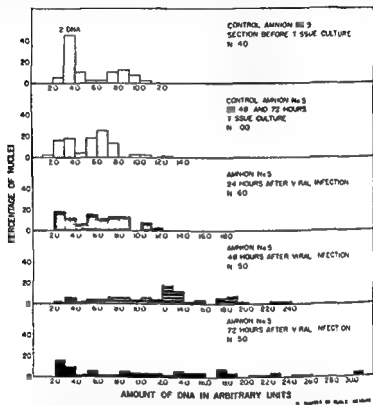


FIGURE 6 Comparison between amounts of DNA (microspectrophotometry) in individual nuclei of human amniotic cells before and after infection with a reovirus Type 7

made up of only diploid that is 2DNA cells, even when undergoing rapid mitosis, will show cells with a DNA content between 2DNA and 4DNA only and not higher.

Perhaps the presence of 4DNA, that is, tetraploid cells in the normal amnion *in situ* explains the frequently reported heteroploid transformation in amniotic tissue cultures.¹³ Since tetraploid cells seem to have a selective advantage in tissue cultures,¹⁴ the heteroploid cells may actually derive from the tetraploid ones.

Comparing the DNA data from the noninfected control amniotic cells with those found at varying time intervals after the viral infection it can be seen

that 24 hours after infection with adenovirus Type 7, the DNA values are essentially the same as those of the noninfected controls. However 48 and 72 hours after viral infection the DNA values are considerably higher with a pronounced variability from cell to cell, resembling closely the DNA values in verruca vulgaris (FIGURE 3). Essentially the same pattern was found in other experiments in which we used amnions from other human deliveries and where the DNA values were determined up to 96 hours after viral infection.

On the basis of the few examples presented here, it appears that when human cells are infected with certain DNA-containing viruses such as verruca vulgaris or adenoviruses, characteristic cytological and cytochemical changes involving the DNA occur in the nuclei of the infected cells. The cytological response and the deviating DNA pattern were found to be very similar in the cases examined irrespective of whether the cells infected were skin cells, amniotic cells, or cancer cells and regardless of whether the cells were still *in situ* in tissues of the human body or were explanted to grow in tissue culture.

Although these data clearly demonstrate the fact that these viruses elicit an abnormal DNA metabolism in the nuclei and disrupt the DNA constancy, the interpretation of the deviating DNA behavior can be only speculative. One of the great difficulties, when attempting to explain the abnormally high DNA values comes from the impossibility of differentiating *in situ* between the intranuclear virus DNA and the host cell DNA by the present methods, including the Feulgen reaction. Therefore at present it cannot be decided whether the large DNA amounts observed are due to multiplying virus DNA or due to an unusual and irregular synthesis of host cell DNA induced by the presence of the virus. That the changes observed may reflect viral propagation is suggested by the similarity of the cytological and cytochemical behavior of cells infected with other viruses such as herpes simplex¹⁸ and the polyhedra virus of the silkworm¹⁹ in which intranuclear viral synthesis is believed to occur. Furthermore the character of these intranuclear changes seems to be almost specific for these virus infected cells and is strikingly different from the appearance of the nuclei of cells engaged in growth mitosis or other metabolic activities and from cells undergoing necrosis and pyknosis.²⁰ As a matter of fact when the microscopist is confronted with the peculiar combination of intranuclear bizarre configuration of DNA masses associated with increase and variability of DNA which cannot be explained on the basis of mitosis and ploidy or polyploidy viral etiology should be considered seriously. Moritz and Leuchtenberger reported such a case in a young man who died of an unexplained respiratory disease.¹⁸ Nearly all the tissues examined contained cells with large nuclei and intranuclear alterations very similar to those found in the virus infected cells just described. In FIGURE 7 the nuclear sizes and the DNA values in the kidney of this man as compared with the values of a normal human kidney are graphed. The similarity of the DNA pattern in the kidney nuclei of this case with those of the verruca or adenovirus infected nuclei is evident. Although we realize of course that the data are not final proof for establishing a viral origin the concept of a possible viral infestation was offered with the suggestion that other pathological tissue processes of unknown etiology representing similar findings also would be examined from a viral point of view.

Summarizing briefly, human cells infected with certain DNA-containing viruses, such as verruca vulgaris or adenovirus, reveal characteristic cytological and cytochemical abnormalities of the DNA pattern. This deviating behavior of the DNA was observed regardless of the type of cells infected (skin, cancer amnion) and irrespective of whether the cells were *in situ* in tissues of the human body or whether they were explanted to grow in tissue culture. The similar response of the cells is especially encouraging, because tissue cultures are

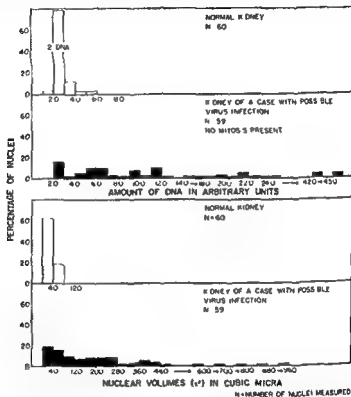


FIGURE 7 Comparison between amounts of DNA (microspectrophotometry) and nuclear sizes of individual cells from normal kidney and kidney from a patient with possible systemic virus disease

excellent biological systems for the correlated cytological and cytochemical exploration of the sequential changes occurring in cells after viral infection. It is hoped that this brief presentation on the quantitative cytochemical investigation of the DNA in virus-infected cells demonstrates the new pathways opened by these cytochemical procedures and stimulates the application of these processes to the elucidation of the many unexplored problems concerning the virus-host cell relationship.

Acknowledgment

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FLUORESCENCE MICROSCOPY OF NUCLEIC ACID CHANGES IN VIRUS INFECTED CELLS

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In the past few years, chemical analysis of purified virus preparations has revealed with increasing certainty that the essential polynucleotide component may be either deoxyribonucleic (DNA) or ribonucleic acid (RNA). It is now established that influenza poliomyelitis, eastern equine encephalomyelitis and West Nile encephalomyelitis viruses contain RNA, vaccinia, Shope papilloma and bacteriophage contain DNA. An investigation of the changes in nucleic acid distribution in virus infected cells thus might be expected to yield information concerning the intracellular site of virus formation and help to elucidate the cytological appearances that are of constant occurrence in some virus infections. During a study of the cytochemical significance of the tissue fluorescence induced by the fluorochrome, acridine orange, Armstrong (1956) found that in thin sections of fixed tissues treated with a dilute solution of the dye, it was possible, with the fluorescence microscope, easily to locate the distribution of nucleic acid-containing material within tissue cells and to distinguish between RNA and DNA. A similar conclusion was reached by Bertalanffy and Bickis, 1956. The advantages of the technique are its simplicity and sensitivity as compared with established methods of staining nucleic acid material and the simultaneous demonstration of both RNA and DNA by a single solution. The sensitivity is particularly well brought out in monolayer tissue culture preparations.

Although all the acridine compounds possess the property of fluorescence the aminoacridines exhibit this phenomenon to a remarkable degree. The brilliant polychromatic fluorescence shown by acridine orange was described by Strügger (1940) who was the first to apply a method of induced fluorescence to the investigation of microbiological problems. Using acridine orange on unfixed material, he claimed to be able to distinguish between living and dead bacterial and plant cells. The differential fluorescence that can be obtained on unfixed material has been interpreted variously by subsequent workers and there is still no unanimity of opinion concerning the results, at the present time the value of the technique for determining the viability of cells has not been established. Investigation of the chemotherapeutic properties of the aminoacridines has indicated that acridine orange and some related compounds have a marked affinity for nucleic acid-containing structures both *in vivo* and *in vitro*, and this specific interaction forms the basis of the technique that has been used for observations on virus infected cells (Armstrong and Niven 1957). Anderson (1957) using a similar technique, has studied the multiplication of bacteriophage in lysogenic bacterial cells induced with ultraviolet radiation.

Since maximal activation of the fluorescence occurs in the blue violet region of the spectrum, quartz optical components are not required, a 250 watt high pressure mercury vapor lamp provides an adequate light source and with a dark ground condenser and suitable filtration, the fluorescent images stand out clearly against a black background. In 3μ sections of fixed animal tissues

structures containing DNA such as nuclear chromatin, show a greenish yellow fluorescence while RNA-containing material gives a bright flame red color. The procedure can be applied equally to smears of cells and tissue culture cells grown on cover slips. Fixation is not critical, only osmium tetroxide and fixatives such as Bouin's fluid containing picric acid inhibit the specific fluorescence. The most important controlling factor is the pH of the staining solution. Within a pH range of 1.5 to 3.5 for acridine orange or 3.5 to 5.0 for acridine orange R the 5 phenyl derivative, differential fluorescence is most striking. The findings have been confirmed by parallel studies of similar material using the Feulgen reaction methyl green pyronine staining nucleic digestion tests and ultraviolet absorption techniques. Since the tissues and cells have been subjected to histological fixation the experimental material is always accompanied by appropriate uninfected controls and, where possible, the microscopic observations have been correlated with virus titration of the material examined. Within the pH range mentioned previously only structures containing acid mucopolysaccharides such as mast cell granules cartilage and elastic tissue exhibit fluorescent properties at all similar to those of nucleic acid-containing elements but identification of these presents no problem.

Members of the pox group of viruses provided interesting material for study. In the case of vaccinia the Guarnieri bodies in corneal epithelium which are known to be Feulgen positive gave a positive DNA fluorescence with the acridine orange technique. In ectromelia (mouse pox) the eosinophilic diagnostic inclusions in squamous epithelium produced by the virus gave a negative result with acridine orange as did the similar inclusions occasionally found in the pancreas and the intestinal epithelium. They also gave a negative Feulgen reaction and did not stain with either pyronine or methyl green. Since early work by Barnard and Elford (1931) had indicated that these inclusions contain virus elementary bodies it is obvious that this problem requires further study. Extensive necrosis occurs in the liver during the course of the disease but although virus can be recovered in high titer from this organ inclusions identical with those of the squamous epithelium do not occur. Many cells however were found to contain spherical bodies showing the typical greenish yellow DNA fluorescence. In cells containing such bodies the nuclei, apart from enlargement and increased prominence and hypertrophy of the nucleoli were at first apparently normal. The RNA fluorescence of the cytoplasmic bodies gradually diminished and eventually disappeared as the cytoplasmic bodies increased in size. Treatment of such sections with 0.01 per cent (w/v) deoxyribonuclease (DNase) at pH 7.4 removed the fluorescence of normal nuclear DNA but did not interfere with the fluorescence of the cytoplasmic DNA in infected cells. Pretreatment with 0.02 per cent pepsin at pH 2.0 for 2 hours at 37° C which did not inhibit the specific DNA fluorescence rendered the cytoplasmic DNA susceptible to subsequent digestion by DNase.

Bearcroft and Jameson (1958) have recently described an infectious disease of rhesus monkeys, transmissible by filtered suspensions, which occurred at Yaba Lagos Nigeria. The disease is characterized by subcutaneous tumorlike lesions that persist for periods up to 10 weeks and then regress. Smears from the cells composing the nodules show the presence of numerous elementary bodies that give a light greenish yellow fluorescence with acridine orange and,

when examined with the electron microscope, are seen to resemble vaccinia in shape and size and in the possession of a pepsin resistant central dense body. The characteristic cells apparently derived from fibroblasts are polygonal in shape with large vesicular nuclei and greatly hypertrophied nucleoli. The cytoplasm contains dense paranuclear bodies of varying shape and size, often coexisting with areas of fine granularity. All these abnormal cytoplasmic structures exhibit a bright DNA fluorescence, and the appearances suggest a series of developmental stages, the earliest being the dense body that becomes transformed into granular material. As in the case of liver cells infected with ectromelia pretreatment with pepsin was necessary before the cytoplasmic DNA could be removed with DNase.

Observations by conventional light microscopy have disclosed profound alterations of nuclear structure in HeLa cells infected with adenovirus (Barski 1956 Boyer *et al* 1957). By combining electron microscopy of ultrathin sections with the Feulgen reaction in alternate thin and thick sections, Bloch *et al* (1957) have localized and determined that the aggregations of virus particles correspond to the Feulgen positive intranuclear material and they consider the virus to be of DNA type. Armstrong and Hopper (in press) studied the cytochemical changes in the HEP 2 line of human cells after infection with adenovirus Type 6 with the acridine orange fluorescence technique and found that enlargement of the nucleolus accompanied by enhanced fluorescence was the earliest detectable change, this was soon followed, however by changes in the pattern of nuclear chromatin. Irregular masses of intranuclear material showing an intense fluorescence of the DNA type associated with a system of vesicles within the nuclear membrane developed, the masses frequently fusing to form a centrally placed body within which the red fluorescing nucleolus could still be made out. This intranuclear DNA is not directly susceptible to the action of DNase but can be removed by the specific enzyme after treatment with pepsin. It thus possesses, like the cytoplasmic DNA already described in other virus infected cells, a protective component that can be removed by proteolytic digestion.

The cytopathogenic changes that occur in tissue culture cells infected with the virus of cytomegalic inclusion disease have been described by Weller *et al* (1957). Through the kindness of Weller it has been possible to make some observations on the development of the intranuclear inclusion bodies that are so characteristic of this condition. Enlargement of the nucleus hypertrophy and increased intensity of fluorescence of the nucleoli, and the appearance of numerous bright greenish yellow fluorescing granules were the earliest changes detected. In all cases the RNA fluorescence of the cytoplasm also increased in intensity particularly around the nucleus. The typical intranuclear inclusion body is formed apparently by the enlargement and fusion of the numerous granules of DNA material seen at an earlier stage. Nuclear vacuolation so characteristic of adenovirus infection does not take place.

The cytopathological effects of West Nile encephalitis have been studied in monolayer cultures of chick fibroblasts. This virus is known to contain RNA as its essential polynucleotide component (Colter *et al* 1957) it was of interest to see if the usual pattern of RNA was changed during virus growth. Enlargement of the nucleus and increased prominence of the nucleolus followed infection.

tion but these reactions were not accompanied by significant alterations of nuclear morphology. The major site of cytological change was in the cytoplasm where the fine RNA granularity was replaced by brighter and coarser RNA fluorescence. Conspicuous masses of bright red material appeared, subsequently, these could be seen close to the cell surfaces attached to long, tenuous processes or lying free. As these cytoplasmic aggregates accumulated nucleolar and nuclear fluorescence gradually diminished. The study of influenza infected tissue cultures has proved to be one of the most interesting applications of the acridine orange technique. Enlargement of the nucleus and of the nucleolus was accompanied by the development of a diffuse red color that filled the nucleus often obscuring all the nuclear structures except the associated chromatin of the nucleoli. The sharp outline of the nuclear membrane became indistinct and acquired a fine beaded appearance suggestive of breaks in continuity. In the cytoplasm, an increased intensity of RNA fluorescence frequently was followed by the development of intense perinuclear fluorescence or by the appearance of tufts of flame red color extending from one or both nuclear poles. The maximal intensity of nuclear fluorescence however was not necessarily accompanied by the most striking cytoplasmic changes. The intranuclear RNA fluorescence was removed by RNase, pretreatment with a proteolytic enzyme was not required.

Summary

In these studies of virus infected cells certain features have been found some of which recall alterations that can occur in physiological conditions and others that so far as is known are not associated with normal cytological activity. The former are illustrated by nuclear and nucleolar enlargement and an increase in cytoplasmic RNA. The latter by qualitative and quantitative changes in the distribution of nucleic acids within the nucleus or cytoplasm or both. It would appear that unique changes in nucleic acid pattern can be initiated following virus infection and these are of 4 kinds: (1) appearance of DNA in the cytoplasm as in pox virus infections, and the spontaneous dispersal of rhesus monkeys which is being investigated; (2) an altered RNA pattern in the cytoplasm as in West Nile encephalitis; (3) the development of RNA in the nucleus quite distinct from the nucleolus as in influenza and (4) the occurrence of DNA aggregates in the nucleus as in adenovirus infections and cytomegalic disease of infants. It may be significant that in all cases where the nucleic acid constituent of a virus agent is known the dominant change has occurred in that nucleic acid type.

It can be argued of course that the changes recorded may be the result of disordered cell metabolism following infection and there is certainly no justification for assuming on the basis of such observations alone that any unusually sited nucleic acid virus nucleic acid that will be incorporated in fully developed infective particles unless the results are supported by collateral evidence from electron microscopy and immunological data such as is given by the Coombs fluorescent antibody technique. In the case of DNA viruses however we have found a relative insusceptibility of the abnormal DNA to the action of DNase in all the material so far examined and subsequent work may reveal some distinguishing quality of the abnormal RNA in cells infected with

RNA viruses. It is necessary to study more viruses, and more information may become available by studying the effect of individual viruses in different types of host cells. The method thus may be capable of giving a hint of the nucleic acid structure of a particular virus.

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CELLULAR CHANGES ASSOCIATED WITH INFECTION OF THE EHRICH ASCITES TUMOR WITH NEWCASTLE DISEASE VIRUS*

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The interaction of Newcastle disease virus (NDV) with Ehrlich ascites tumor (EAT) cells of the mouse has been the subject of several reports published within the past few years.¹⁻⁶ These studies have employed NDV adapted to growth in EAT cells referred to as EAT adapted NDV as well as ordinary laboratory strains propagated by serial passage in eggs referred to as chick embryo adapted NDV. The EAT adapted virus is able to replicate infectious virus in EAT cells,¹⁻³ while chick embryo adapted NDV is not,⁴⁻⁶ although the latter is able to infect and destroy EAT cells. This paper is limited to a consideration of the morphology of Ehrlich ascites tumor cells infected with chick embryo adapted Newcastle disease virus.

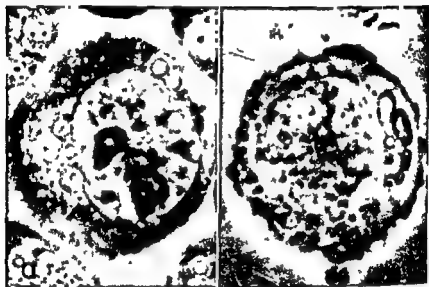
In a recent study of the interaction of chick embryo adapted NDV with EAT cells the fluorescent antibody technique was employed to demonstrate what appeared to be an exceptionally rudimentary form of incomplete virus.⁷ The results of this study indicated that infection of EAT cells with this virus resulted in the intracytoplasmic synthesis of a component that had the ability to combine specifically with fluorescein labeled anti NDV serum but that had no detectable hemagglutinin activity and no measurable complement fixing ability and was not infective for embryonated eggs. This observation suggested the interesting possibility that the newly synthesized component might be only a small fragment of the intact virus particle possibly a single molecular species. Replication of a more complex form of virus was by no means ruled out however for this reason morphologic study of the Ehrlich NDV system was carried out in an attempt to determine whether the newly synthesized antigen was as simple morphologically as it was functionally or whether it was associated with a unit more nearly resembling the intact infective virus as is the case with incomplete influenza virus produced by passage in the mouse brain or by serial and lured passage in the allantoic cavity of the chick embryo.⁸

Our approach was threefold and employed the following procedures phase microscopy carried out on fresh unfixed wet preparation conventional light microscopy carried out on paraffin embedded cells stained with hematoxylin and eosin Giemsa pyronine-methyl green Feulgen, and methenamine silver and electron microscopy of material that had been fixed in osmium tetroxide and embedded in methacrylate. A detailed description of materials and techniques employed has been published elsewhere.⁹

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Phase Microscopy and Light Microscopy

A typical infection experiment is observed with the phase microscope occupied a total of less than 15 hours. Washed Ehrlich ascites cells, harvested 6 days after inoculation, were sedimented by low speed centrifugation and re-suspended in a partially purified preparation of NDV. The concentration of virus and cells was adjusted to give a ratio of from 50 to 1000 infective particles per cell. The mixture of virus and cells was then placed in an ice bath for about 30 min. in order to allow adsorption to take place. Following adsorption aliquots of the mixture were injected into the peritoneal cavity of



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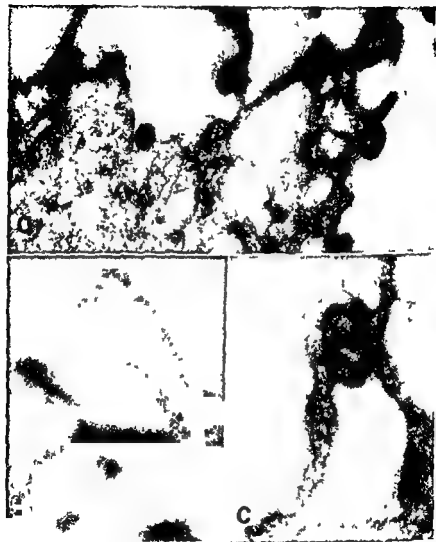
Experimental Medicine

fresh mice and the infection allowed to proceed, samples being removed at
microscope
typical uninfected
fine hairlike pseu
dopodia averaging about $1\ \mu$ in length a well-defined nucleus with irregularly
clumped chromatin and two readily distinguishable cytoplasmic components
mitochondria that appear as short dark rodlets and brilliantly refractile ob
jects that have been demonstrated to contain lipid. Infected cells, in the
time interval 0 to 4
the normal cell shows
infection a new cla
objects gradually increase in size and number until virtually every cell in
infected population is involved. Initially, they usually occupy a juxtanuclear

position and are neither as dark as mitochondria nor as refractile as lipid droplets. Their contours are usually smoothly lobulated and little internal structure can be visualized at the resolution offered by the phase microscope. Numerous variations in shape are usually seen. Some are approximately spherical and measure from 1 to 4 μ in diameter. Still others are ellipsoidal, and occasional forms are greatly elongated measuring up to 10 μ or more in length. The ultimate fate of infected cells is lysed. With the virus cell ratios employed in this experiment the lytic process begins 6 to 8 hours following infection usually by 12 to 15 hours the vast majority of the infected cells has undergone disintegration. The nuclei of the disintegrating cells usually showed little change until after complete dispersion of the cytoplasmic components when a gradual process of pyknosis set in ultimately resulting in small dark irregular masses of nuclear material about the size of a red blood cell. The process of disintegration as viewed with the phase microscope was never a sudden fulminant event but usually occupied an interval of 15 to 30 minutes. The extended duration of the dissolution process seemed to be due primarily to the presence in the vicinity of the nucleus of an invisible but highly viscous gel that hindered the centrifugal motion of the cytoplasmic material. Conventional light microscopy performed on paraffin embedded material failed to reveal clear-cut evidence of the cytoplasmic bodies demonstrated so clearly with phase contrast.

Electron Microscopy

Cells examined after adsorption of virus but prior to placement in the peritoneal cavity of the mouse differ morphologically from control cells in only one particular adsorbed particles tentatively identified as Newcastle disease virus may be seen on and between the pseudopodia (FIGURE 2a, b and c). The overall morphology of these particles is quite uniform, although there is some variation in size. These particles can be seen to have a double limiting membrane each element of which measures approximately 30 to 40 \AA in thickness enclosing several small dense regions that are rather uniform in size and measure about 110 \AA in diameter. The identification of these particles as Newcastle disease virus is supported by several considerations. First they are denser and more uniform with which they might be confused. Second their sections of pseudopodia agree roughly assuming a spherical shape with other estimates of the size of NDV. Third similar particles have been identified in electron micrographs of NDV agglutinated chicken red blood cells but have never been seen on uninfected Ehrlich cells or on chicken erythrocytes suspended either in various buffers or in uninfected chick embryo allantoic fluid. Finally the particles disappear from the surface of Ehrlich cells within 30 min after immersion with fluorescent antibody studies which indicate that virus adsorbed to the surface of EAT cells is initially visible as a bright peripheral ring of fluorescence that disappears after incubation for 15 to 30 min either *in vivo* or *in vitro*. Disappearance is not due to elution at least of hemagglutinating particles. The mode of penetration of the plasma membrane by the virus



Experiments in sea water

It is possible on the whole that a hint of what may be occurring here, which there is particle at adjacent cell wall. Substantiation of this point would suggest that the inner portion of the virus may enter the cell prior to, and perhaps separate from, the outer coat of

It has been washed at 17 of

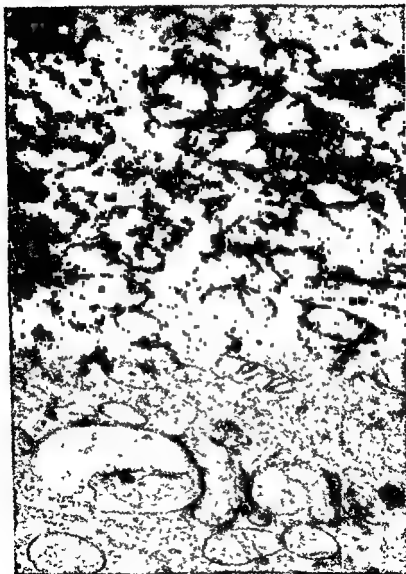


FIGURE 3 Electron micrograph of a portion of the cytoplasm of an Ehrlich ascites tumor

the virus particle. Cells studied at intervals of 30 min and 2 hours following infection no longer have the adribed surface particles just mentioned and are indistinguishable from control cells. The cytoplasmic inclusions that appear following infection with Newcastle disease virus are first clearly seen after 4 hours incubation in the peritoneal cavity of the mouse (FIGURE 3). There



after they increase rapidly in size and number, as FIGURE 4, 10½ hours after infection, illustrates. No nuclear involvement has been seen in any of the preparations studied. Although the inclusions exhibit individual variations in appearance, their over all morphology has several characteristic features



FIGURE 5. Electron micrograph of an Ehrlich ascites tumor cell 10½ hours after infection with NDV illustrating the close association between Golgi material and the shell of the inclusions. X39,000. Reproduced by permission from *The Journal of Experimental Medicine*.⁸

illustrated in FIGURES 5 and 6. Usually they are elliptical or circular in profile and may measure up to several microns in diameter. They have a limiting shell composed of a variable number of laminae measuring 4 to 8 mμ in width and usually associated with small vesicles. The limiting shell encloses a core



FIGURE 11 Electron micrograph of an inclusion adjacent to the nucleus of an Ehrlich ascites tumor cell.

later for example at 8 or 10 hours after infection usually had at least a dozen layers and occasionally many more. The vesicles associated with the laminae have the appearance of transversely sectioned tubules that resemble and are frequently continuous with adjacent clusters of vesicles that can be identified as elements of the smooth surfaced reticulum or Golgi material. Frequently 1 of the laminae can be seen to split into 2 thinner lamellae that are continuous



FIGURE 7 Electron micrograph of an Ehrlich ascites tumor cell 10½ hours after infection with Newcastle disease virus. At this stage of infection approximately 5 to 10 per cent of infected cells revealed varying degrees of vacuolization and mitochondrial swelling as depicted in this figure. This cell is identified as an EAT cell by the presence of two characteristic intracellular particles of EAT at the extreme upper left (arrow). X22,500. Reproduced by permission from *The Journal of Experimental Medicine*.

with the wall of 1 of the adjacent vesicles. An example of this may be seen at the bottom of FIGURE 5. This observation strongly suggests that the lamellar portion of the shell may be formed by a process of collapse and fusion of vesicles of the smooth surfaced reticulum. Considerable variation in the morphology of the internal granular material was noted among different inclusions; however the granules within any particular inclusion displayed a somewhat greater uniformity. No correlation was demonstrated between the

size of the granules and the size of the inclusion, or the number of laminae comprising its shell. Few if any significant changes were seen in the cytoplasmic organelles during the early stages of infection. However, in the end stages of infection, marked vacuolization of the vesicular components of the

numbers of them were seen extracellularly at a time when cytolysis was progressing rapidly.

Comments

In answer to the initial question concerning the morphology of the incomplete viral antigen previously demonstrated by fluorescent antibody studies, these results would seem to suggest strongly that the newly synthesized antigenic units are much smaller than the native virus particles perhaps no larger than 10 to 14 μ in diameter, and that they may, in fact, be identical with the granular material present in the core of the cytoplasmic inclusions. This antigenic material has been thought of as a subunit of the intact virus. However the possibility that the antigen represents a nonviral, noncellular protein associated with the infection process must also be considered. Any hypothesis linking the granules that comprise the core of the inclusions with the fluorescent antibody binding antigen is, of course, highly conjectural, particularly since

to be tested further. First, no large particles bearing resemblance to the native virus particles were ever seen in any of the sections examined. Second the particles within the inclusions exhibit much greater variation in size than do the cytoplasmic granules. Third, no forms demonstrating continuity between the granular material of the inclusion and the cytoplasm of the cell have been seen among the many hundreds of inclusions studied in the electron microscope. Finally, it is not unreasonable to assume that the newly formed antigenic units, if they are visible at all, most likely would be associated with the newly formed morphologic entity, that is the cytoplasmic inclusion. Another possibility, of course is that the new synthesized antigenic material is associated with the shell of the inclusion rather than with the granular material in its core. This seems improbable, however, in view of the demonstrated close association of the shell with the Golgi material of the Ehrlich cell suggesting that the shell is of cytoplasmic origin.

The general significance of the cytoplasmic inclusions here demonstrated remains speculative. Even though these inclusions seemed to be unquestionably related in this particular system to infection of cell with virus it is unlikely that they are unique to this particular cell system for two reasons. First, although none of the control preparations in the present set of experiments contained structures bearing any resemblance to the inclusions that developed in infected cells such structures have been seen on rare occasions in our laboratory in other preparations of uninfected Ehrlich cells. Second similar

Adams & Prince Infected Ehrlich Ascites Tumor

appearing laminated structure, have been described by other investigators in other virus-cell systems.¹⁹ These considerations suggest the possibility that this type of inclusion is simply a particular example of a more generalized reactive phenomenon on the part of Ehrlich cells and possibly of other cell types as well. If this is a valid generalization one might then envision a new and unsuspected function of the Golgi apparatus that is participation in a type of intracellular defense mechanism. The possibility of such a function is certainly reasonable on theoretical grounds and receives some additional support from recent and rapidly developing evidence that the physiological state of the host cell is of paramount importance in determining not only whether infection occurs but to what extent the invading virus is successful in commandeering intracellular systems for the purposes of self duplication. Two examples that lend support to this idea are first a study of VDI in infected L cells by Wilson²⁰ which demonstrated the fact that the quantity of infective virus produced by infected cells can be increased by injuring the cell, (either by decreasing incubation temperature or by treating them with sublethal concentrations of cyanide) and second the observation that chick embryo-adapted VDI is able to undergo complete replication in Ehrlich ascites tumor cells, only in tissue culture under suboptimal nutritional conditions.²¹ It remains to be seen whether the presence or activity of the laminated inclusion plays any role in these functions of the cell that are able to limit the process of virus multiplication to some form of partial replication.

Summary

The morphologic changes occurring in Ehrlich ascites tumor cells infected with chick embryo adapted Newcastle disease virus were studied with phase microscope, conventional light microscope and electron microscope. Intracytoplasmic inclusions appeared 2 to 4 hours following infection and progressively increased in size and number until cytolysis occurred. Significant alterations in mitochondria and other cell organelles did not appear during the initial period of inclusion development but were seen shortly before cytolysis began (8 to 10 hours after infection). The inclusions were composed of a laminated shell possibly derived from the agranular reticulum of the host cell, surrounding a central portion containing dense particles that measured 3 to 14 m μ in diameter. The identity of these particles and a possible function of the inclusions are discussed.

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size of the granules and the size of the inclusion, or the number of laminae comprising its shell. Few if any significant changes were seen in the cytoplasmic organelles during the early stages of infection. However, in the end stages of infection, marked vacuolization of the vesicular components of the cytoplasm occurred and at the same time, the cytoplasmic inclusions appeared to approach the plasma membrane (FIGURE 7). The inclusions appeared to be at least somewhat resistant to the process of dissolution since increasing numbers of them were seen extracellularly at a time when cytolysis was progressing rapidly.

Comments

In answer to the initial question concerning the morphology of the incomplete viral antigen previously demonstrated by fluorescent antibody studies, these results would seem to suggest strongly that the newly synthesized antigenic units are much smaller than the native virus particles, perhaps no larger than 10 to 14 μ in diameter, and that they may, in fact, be identical with the granular material present in the core of the cytoplasmic inclusions. This antigenic material has been thought of as a subunit of the intact virus. However, the possibility that the antigen represents a nonviral, noncellular protein associated with the infection process must also be considered. Any hypothesis linking the granules that comprise the core of the inclusions with the fluorescent antibody binding antigen is, of course, highly conjectural particularly since these particles are within the size range of the RNA-containing cytoplasmic granules and may represent nothing more than isolated islands of cytoplasm. Several considerations, however, do recommend this as a working hypothesis to be tested further. First, no large particles bearing resemblance to the native virus particles were ever seen in any of the sections examined. Second the particles within the inclusions exhibit much greater variation in size than do the cytoplasmic granules. Third, no forms demonstrating continuity between the granular material of the inclusion and the cytoplasm of the cell have been seen among the many hundreds of inclusions studied in the electron microscope. Finally, it is not unreasonable to assume that the newly formed antigenic units, if they are visible at all, most likely would be associated with the newly formed morphologic entity, that is the cytoplasmic inclusion. Another possibility, of course is that the new synthesized antigenic material is associated with the shell of the inclusion rather than with the granular material in its core. This seems improbable, however, in view of the demonstrated close association of the shell with the Golgi material of the Ehrlich cell suggesting that the shell is of cytoplasmic origin.

The general significance of the cytoplasmic inclusions here demonstrated remains speculative. Even though these inclusions seemed to be unquestionably related in this particular system to infection of cell with virus, it is unlikely that they are unique to this particular cell system for two reasons. First, the present state of experiments developed in our laboratory in other preparations of uninfected Ehrlich cells. Second, similar

appearing laminated structures have been described by other investigators in other virus-cell systems. These considerations suggest the possibility that this type of inclusion is simply a particular example of a more generalized reticulate phenomenon on the part of Ehrlich ascites cells and possibly of other cell types as well. If this is a valid generalization one might then envision a new and unsuspected function of the Golgi apparatus that is participation in a type of intracellular defense mechanism. The possibility of such a function is certainly reasonable on theoretical grounds and receives some additional support from recent and rapidly developing evidence that the physiological state of the host cell is of paramount importance in determining not only whether infection occurs but to what extent the invading virus is successful in commandeering intracellular systems for the purposes of self duplication. Two examples that lend support to this idea are first a study of NDV in infected L cells by Wiktor¹ which demonstrated the fact that the quantity of infective virus produced by infected cells can be increased by injuring the cells (either by decreasing in vitro temperature or by treating them with sublethal concentrations of cyanide) and second the observation that chick embryo adapted NDV is able to undergo complete replication in Ehrlich ascites tumor cells only in tissue culture under suboptimal nutritional conditions.² It remains to be seen whether the presence or activity of the laminated inclusion plays any role in these functions or as activity of the laminated inclusion process of virus multiplication to some form of partial replication.

Summary

The morphologic changes occurring in Ehrlich ascites tumor cells infected with chick embryo adapted Newcastle disease virus were studied with phase microscope, conventional light microscope and electron microscope. Initially cytoplasmic inclusions appeared 2 to 4 hours following infection and progressively increased in size and number until cytolysis occurred and progressive alterations in mitochondria and other cell organelles did not appear during the initial period of inclusion development but were seen shortly before cytolysis began (8 to 10 hours after infection). The inclusions were composed of a laminated shell possibly derived from the agranular reticulum of the EAT cell, surrounding a central portion containing dense particles that measured 3 to 14 mμ in diameter. The identity of these particles and a possible function of the inclusions are discussed.

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Ph D Thesis Univ

Washington, Seattle, Wash

CYTOPATHOLOGY OF VIRUS INFECTED TUMOR CELLS

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Introduction

Thirty six years ago Levaditi and Nicolau demonstrated the multiplication of herpes simplex and neurovaccinia viruses in transplantable carcinomata of mice.^{1,2} Growth of neurovaccinia virus was accompanied by oncolysis.³ Since then the oncolytic effect of virus infection has been demonstrated repeatedly.⁴⁻⁶ The possible value of viruses in the treatment of cancer and, in particular, of viruses adapted to grow in tumors has not been assessed fully. The disappointing results of chemotherapy⁷ and, by comparison, the remarkable oncolytic action of many viruses^{1-3,7-11} would seem to justify further research in this field.

The implication of viruses in the initiation, promotion, and causation of some forms of neoplasia is well known. Latent virus infection in tumors has been suspected¹² and demonstrated.¹³ Thus, apart from the need to acquire fundamental knowledge of the virus-cell relationship, the study of virus-infected tumor cells may facilitate an understanding of the relationship between virus infection and neoplasia.

Certain patterns of cytopathology become apparent in the study of different types of tumor cells infected with viruses. Some of these patterns simulate closely disorders of the cell resulting from other causes. In discussing the cytopathology of various virus infections an attempt will be made to compare and contrast cellular degeneration that is not attributable to known virus infection.

Cytochemistry of Uninfected Ehrlich Ascites Tumor Cells

The morphology of an uninfected Ehrlich ascites tumor stained by a modified bone marrow strain¹⁴ is shown in FIGURE 1. The nucleus contains several prominent nucleoli, inside many of which there are fine vacuoles. In addition to the chromatin and nucleoli, the nucleus also contains amorphous, finely granular parachromatin¹⁵ (or nuclear "sap"), which is acidophilic in this preparation. In old tumors, such as the one shown in FIGURE 1, the chromatin and the parachromatin of an occasional cell are margined and one or more acidophilic bodies or inclusions* are formed. The nature of these bodies can be elucidated in preparations stained by the toluidine blue molybdate (TBM) method.¹⁷ FIGURES 2a, b, c, and 3a. It may be emphasized that all the staining in FIGURES 2a, b, c, and 3a is due to ribonucleoprotein (RNA protein). In preparations stained by the TBM method, the RNA protein of the parachromatin is stained purple and that of the nucleolus, green. Many of the nucleoli contain metachromatic RNA protein bodies or nucleolini,¹⁸ as shown in FIGURE 2b. The nucleolini appear to correspond to the vacuoles seen in other preparations (compare this with FIGURE 1). A cell showing margination of the parachromatin with the formation of inclusions containing RNA is shown in FIGURE 2c.

* The term inclusion or inclusion body is used throughout this paper to refer to something other than the normal organelles, that is included in the cell and that need not be a *de novo* formation.



FIGURE 1 Smear of Ehrlich ascites tumor stained by a modification of Barrett stain¹¹. Note the minute vacuoles (V) in a nucleolus. Two acrophilic bodies (B) are present in a degenerating cell. $\times 1600$.



Under the microscope it may be seen that the large inclusions consist of a homogeneous green staining center, derived from the nucleolus, covered by purple parachromatin granules. In other degenerating cells smaller inclusions appear to be entirely formed by condensation of the parachromatin (FIGURE 3a). A study of old ascites tumors reveals that these cells eventually disintegrate by karyorrhexis and that the fragments are ingested by macrophages.

The cytoplasm of the Ehrlich ascites tumor cell contains three kinds of lipid particles.¹⁹ A variable number of large lipid droplets stain red with Nile blue sulfate. These are found in increased numbers in degenerating cells in old tumors and usually in virus infected cells.^{7, 8, 10} In addition finely granular or amorphous acidic periodic acid Schiff positive (PAS positive) lipid is found chiefly in the nuclear border area of the cytoplasm (FIGURE 3b). In smears fixed in formal sublimate this material gives a positive acid hematin reaction for phospholipid¹⁹ it is also stained by the Kolatchew technique for demonstrating the Golgi apparatus²⁰ and is present in the region of the cell where the Golgi apparatus is demonstrable by electron microcopy (FIGURE 4). This phospholipid component will be called the Golgi lipid. The third lipid-containing structure in the cytoplasm is the mitochondrion. The mitochondria are more regular in size than the Golgi lipid particles and are distributed more evenly throughout the cytoplasm (FIGURE 5). In the degenerating cells of old tumors

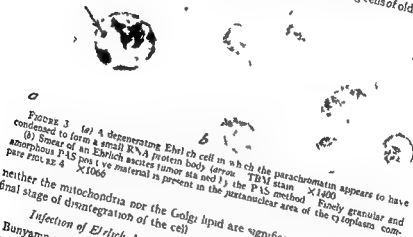


FIGURE 3 (a) A degenerating Ehrlich cell in which the parachromatin appears to have condensed to form a small RNA protein body (arrow). TBM stain $\times 1400$. (b) Smear of an Ehrlich ascites tumor stained by the PAS method. Finely granular and amorphous PAS positive material is present in the paranuclear area of the cytoplasm (compare FIGURE 4 $\times 1066$).

neither the mitochondria nor the Golgi lipid are significantly altered until the final stage of disintegration of the cell.

Infection of Ehrlich Ascites Tumor Cells with Bunyamwera Virus

Bunyamwera virus infection rapidly destroys the Ehrlich ascites tumor.⁷ The earliest change detectable in the virus infected cell consists of margination of the chromatin and the parachromatin. The nucleus is enlarged slightly and the chromatin forms a fine net enclosing the shrunken nucleoli. The nucleoli not only shrink but fragment so that as many as ten or more may be seen in a single cell (FIGURE 6a). The parachromatin appears to condense and possibly to increase in size of the uninfected tumor. Their acidophilic inclusions are cytochemically indistinguishable from the RNA protein inclusions in degenerating cells and is therefore attributable to the presence of protein bound amino groups. The inclusion contains no DNA stainable by the Feulgen reaction but RNA can be demonstrated in them by the toluidine blue methylate method.⁷ The ultimate fate of the infected cells is depicted in FIGURE 7.



FIGURE 4 An electron micrograph of an Ehrlich ascites tumor cell showing the aggregation of Golgi membranes and vesicles in the nuclear hof of the cytoplasm. This photograph was provided through the courtesy of Darlene C. Brindley. $\times 26,000$



FIGURE 5 Smear of the Ehrlich ascites tumor stained by the toluidine blue methionine. $\times 1356$

FIGURE 7 The RNA protein inclusions are not extruded into the cytoplasm until the nucleus has disintegrated. There is some increase in the neutral lipid droplets in the cytoplasm of cells showing nuclear changes but the Golgi lipid and the mitochondria are not altered detectably until the cell disintegrates.



FIGURE 7 Final nuclear disintegration of Ehrlich ascites tumor cells infected with Bunyamwera virus, modified Barlett stain. $\times 1400$

Infection of the Ehrlich Ascites Tumor Cell with Newcastle Disease Virus

Newcastle disease virus (NDV) has been adapted to infect and to destroy the Ehrlich ascites tumor cell. The virus was thawed at 37°C . After one egg passage the first egg passage material was again inoculated into eggs and then into the Ehrlich ascites tumor *in vivo* by methods previously described¹². In each instance approximately 10 million tumor cells were inoculated intraperitoneally into Swiss mice followed 4 to 5 days later by inoculation of virus by the same route. Smears of the peritoneal fluid were prepared daily, stained by Leishman's

method, and examined for evidence of oncolysis.²² The fluid was usually harvested and titrated²³ in eggs 72 hours after inoculation of virus. After 2 passages in the tumor, the virus was again inoculated into eggs. This egg-passaged material was then passed through the tumor *in vivo* for 6 passages, at the end of which 2 per cent of tumor cells in karyorrhexis were found in the smears, the LD₅₀ titer of this fluid in eggs was $10^{-5.20}$. After 2 further passages in the tumor the LD₅₀ titer of the fluid in eggs was greater than 10^{-5} and, in 3 different experiments, 16, 17, and 22 per cent of cells in karyorrhexis were noted in smears prepared 24 to 72 hours after infection (FIGURE 8). In smears of un inoculated control mice the incidence of karyorrhexis was never more than 1 per cent. Thus, after a total of 2 egg passages and 10 passages through the Ehrlich

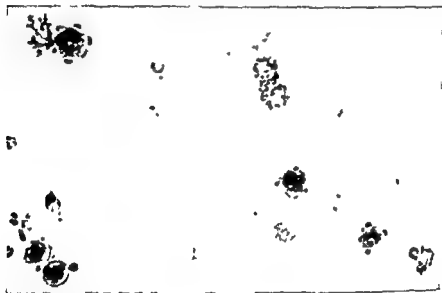


FIGURE 8 Smear of Ehrlich ascites tumor 72 hours after inoculation of the readapted NDV. Five cells in karyorrhexis and five cells with intact nuclei are present. Leishman stain. $\times 340$.

tumor *in vivo*, infection with NDV produced a marked cytopathogenic effect on the Ehrlich tumor.

The cytological changes in Ehrlich cells infected *in vivo* with the adapted NDV are similar to those described by Adams and Prince who used the unadapted virus.²⁴ The first change detectable under the phase microscope 24 hours after inoculation of virus is the development of numerous inclusions in the Golgi zone of the cytoplasm (FIGURE 9a and b). The inclusions are less refractile than the bright neutral lipid globules of the uninfected tumor (FIGURE 10), they are often vacuolated and assume very irregular shapes (FIGURE 9a and b). Cytochemical tests reveal that the inclusions consist of doubly refractile PAS positive acidic phospholipid (FIGURES 11 and 12). They are therefore, chemically similar to the Golgi lipid granules. On the other hand they are larger, often elongated and vacuolated and, in fixed material they exhibit Maltese-cross birefringence.

In cells infected with NDV and stained by the toluidine blue molybdate method the Golgi region which is the site of the phospholipid inclusions is clear or coarsely vacuolated (FIGURES 13a and b, 14a and b) The nucleus is

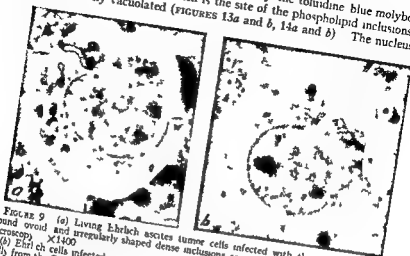


FIGURE 9 (a) Living Ehrlich ascites tumor cells infected with the readjusted NDV. Round ovoid and irregularly shaped dense inclusions are present in the cytoplasm phase microscopy $\times 1400$ (b) Ehrlich cells infected with NDV. Elongated and club shaped inclusions project radially from the Golgi area of the cytoplasm phase microscopy $\times 1400$

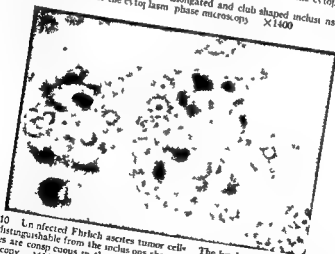


FIGURE 10 An infected Ehrlich ascites tumor cell. The bright refractile lipid droplets are readily distinguishable from the inclusions shown in FIGURES 9b and 10. Numerous minute vacuoles are conspicuous in the Golgi area between the two nuclei of the larger cell phase microscopy $\times 1400$

frequently indented and eccentric. The nucleoli are enlarged and the nucleolus is very prominent. The finely granular parachromatin frequently is concentrated in the indentation of the nucleus adjacent to the Golgi zone (FIGURES 13a and b and 14b). Minute buds of parachromatin often project from the nucleus in this region and as much as 30 per cent of infected tumor cells contain granules of RNA in the nuclear hof (FIGURES 13b, 14a and b). Only a small

proportion of these nuclear extrusions is associated with any DNA that can be detected by the Feulgen method. The dinitrofluorobenzene and ninhydrin-Schiff tests²³ indicate that protein is associated with the RNA. Therefore



FIGURE 11. Inclusions in Ehrlich ascites cells infected with NDV. PAS stain. $\times 1600$.



FIGURE 12. (a) Uninfected and (b) NDV infected Ehrlich ascites tumor cells. Note scanty large lipid droplets and finely granular Golgi lipid in the uninfected cells. Prominent round and ovoid lipid inclusions are stained in the Golgi zone of the infected cells. Sudan black B. $\times 1600$.

RNA protein, which is indistinguishable from the parachromatin, is frequently extruded from the nucleus into the Golgi zone of the cytoplasm. Comparison of the toluidine blue molybdate preparations with the phase microscope obser-

(FIGURE 9b) The presence of RNA protein of nuclear and possibly, of viral origin in the inclusions is consistent with the electron microscope observations of Adams and Prince²⁴ The possibility that the inclusions may contain virus is suggested also by the demonstration of specific viral antigen in them²⁵

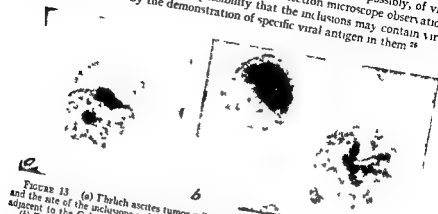


FIGURE 13 (a) Ehrlich ascites tumor cell infected with NDV. The nucleus is eccentric and the site of the inclusions is faintly vacuolated adjacent to the Golgi zone. TBM stain, X1600. Note the aggregation of parachromatin. (b) Two Ehrlich cells infected with NDV. RNA protein bodies are stained in the Golgi area and club-shaped processes of RNA protein protrude from the nucleus into the Golgi zone. TBM stain, X1300.

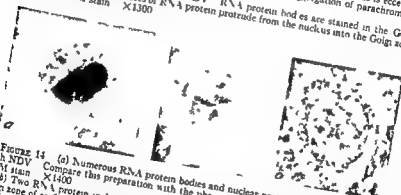


FIGURE 14 (a) Numerous RNA protein bodies and nuclear protrusions in a cell infected with NDV. Compare this preparation with the phase microscope photograph (FIGURE 9b). TBM stain, X1400. (b) Two RNA protein inclusions and a club shaped protrusion from the nucleus into the Golgi zone of an Ehrlich cell infected with NDV. Note the concentration of metachromatic (dark staining) parachromatin adjacent to the Golgi area. TBM stain, X1130. (c) Uninfected rat HeLa tumor cell. The round and ovoid mitochondria are evenly distributed throughout the cytoplasm. phase microscopy, X960.

The development of the cytoplasmic inclusions is accompanied by an increase in the number and size of the neutral fat droplets of the cell. The mitochondria appear to be displaced by the accumulation of inclusions in the Golgi zone but they are not significantly altered until the final disintegration of the cell. Destruction of the cell is completed by nuclear shrinkage followed by karyorrhexis and disruption of the cytoplasm. Inclusions, nuclear fragments, and neutral fat droplets are liberated and frequently are seen inside the macrophages that replace the tumor cells in the ascitic fluid.

Infection of Rat HeLa Cells with Coxsackie B3 Virus

Inoculation of HeLa tissue cultures into the peritoneum of cortisone-treated irradiated rats produces large tumors.^{11, 12} The growth of these tumors is not significantly affected by infection with Coxsackie B3 virus.^{11, 12} After several



FIGURE 15 Smear of uninfected rat HeLa tumor cells. The Golgi lipid consists of flattened vesicles and minute granules. Sudan black B. $\times 1700$



FIGURE 16 (a) Prominent acidophilic inclusion in an uninfected rat HeLa cell. modified Barrett stain. $\times 1400$

(b) Circumscribed accumulation of fine granular material in the Golgi area of a rat HeLa cell infected with Coxsackie B3 virus. The nucleus (arrow) is shrunken. phase microscopy. $\times 1070$

(c) Smear of a rat HeLa cell infected with Coxsackie B3 virus. The pyknotic nucleus is displaced by a circumscribed mass of sudanophilic material. Sudan black B and carmalum stain. $\times 2465$

passages of this virus through the rat HeLa tumor, a striking oncolytic effect is observed.¹² The cytological changes leading to destruction of the rat HeLa cell resemble those found in the Ehrlich cell infected with Newcastle virus. Before discussing the infected rat HeLa cell a few aspects of the morphology and cytochemistry of the uninfected cell must be emphasized.

The appearance of the uninfected rat HeLa cell under the phase microscope is shown in FIGURE 14c. There are numerous round and ovoid mitochondria in the cytoplasm. The Golgi lipid gives a positive acid hematin reaction¹³ and



FIGURE 17. Kolatchew Golgi stain of an uninfected rat HeLa tumor. The Golgi apparatus is very minute in the healthy cells (*right*) and there is no significant increase in osmophilic material in the degenerating cells (*left*). $\times 735$.

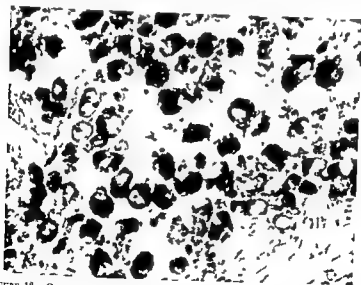
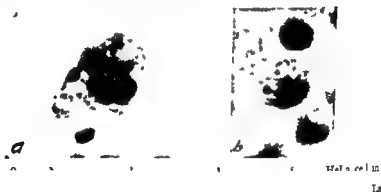


FIGURE 18. Conspicuous increase in osmophilic material in rat HeLa cells infected with Coxsackie B3 virus. Kolatchew Golgi preparation. $\times 735$.

therefore contains phospholipid; it stains with Sudan black (FIGURE 15) and by the PAS method. FIGURE 16a shows an intranuclear RNA protein inclusion in an uninfected rat HeLa cell. These inclusions are not uncommon in the nuclei of HeLa cells at the edge of areas of ischemic necrosis.

Twenty-four hours after inoculation of the adapted Coxsackie B3 virus, char-

acteristic changes appear in the rat HeLa cells (FIGURE 16b). Under the phase microscope a circumscribed accumulation of granular material is seen in the Golgi zone of cells with pyknotic nuclei. The mitochondria are included in this material and the rest of the cytoplasm appears to be structureless (FIGURE 16b). In the more advanced stages of degeneration the mitochondria can no longer be recognized and the pyknotic nucleus is displaced or even indented by a dense vacuolated mass in the cytoplasm. The cytochemical and tinctorial properties of the cytoplasmic granular material are similar to those of the Golgi lipid* (FIGURES 16c, 17, 18). In addition to the lipid material, numerous very minute granules of ribonucleoprotein are often found in the Golgi zone* (FIGURE 19a and b). The nuclei of cells containing accumulations of Golgi lipid are shrunken and pyknotic and occasionally, minute spikelike projections into the Golgi zone are seen (FIGURE 19b). It is probable, therefore, that the RNA protein granules in the virus infected cells are derived from the nucleus in the same way as those found in Ehrlich cells infected with NDV.



Destruction of the nuclei of rat HeLa cells infected with Coxsackie virus is completed by karyolysis and fragmentation of the nucleus into dustlike particles. The cytoplasm persists *in situ* for several days and finally is removed by neutrophil granulocytes and macrophages.

Other Virus Infections of Tumor Cells Characterized by Increase in Golgi Type Lipid

Infection of the Ehrlich ascites tumor by Mengo, West Nile, and Anopheles A viruses results in oncolysis.¹⁰ Each of these viruses produces an increase in the Golgi lipid similar to that observed with Coxsackie B3 and Newcastle disease virus infections. FIGURE 20 illustrates the great increase in this material following infection with West Nile virus.

In addition to changes in the Golgi lipid, well defined acidophilic structureless ovoid zones develop in the cytoplasm of practically every Ehrlich tumor cell

from the Golgi

the virus induced necrotic regions described by Friedlaender *et al*²⁷ in electron micrographs of this material. They have not been observed in any other virus infected or uninfected cell. Infection of the RPL-12 lymphoma of the chicken with St. Louis encephalitis virus produces an increase in the Golgi lipid⁹. In smear preparations or frozen

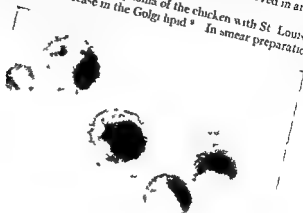


FIGURE 20 Striking increase in PAS positive lipid in the Golgi zone of Ehrlich ascites cells infected with West Nile virus (compare FIGURE 3b) PAS stain X1600

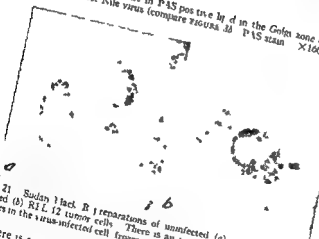


FIGURE 21 Sudan black B preparations of uninfected (a) and St. Louis encephalitis virus infected (b) RPL-12 tumor cells. There is an increase in the number and size of the lipid particles in the virus-infected cell frozen sections X2000

sections there is an accumulation of PAS positive sudanophilic granules in the Golgi zone (FIGURE 21). This material persists in paraffin sections and fuses to simulate a faintly acidophilic inclusion body (FIGURE 22a). These inclusions are readily stained by the PAS method⁹ as shown in FIGURE 22b. Infection of the chicken lymphoma with St. Louis encephalitis virus does not in itself lead to oncolysis^{9, 10}. When this tumor is transplanted in the mouse the characteristic Golgi changes develop. Oncolysis is completed by phagocytosis of the abnormal cells. When the tumor is transplanted into the brain

the same changes develop in the Golgi lipid, but phagocytosis is deficient. The tumor cells continue to proliferate and kill the host.⁶ Mitotic division of cells with hypertrophied Golgi lipid is however defective. Analysis of mitosis in the infected tumor reveals a significant increase in cells in metaphase a decrease in those in telophase and an increase in binucleate forms (TABLE I). This indicates some degree of metaphase arrest together with defective cytoplasmic division. The accumulation of lipid in the infected cells frequently displaces and even indents the nucleus. It is possible therefore that the pres-



TABLE I
ANALYSIS OF MITOSIS AND BINUCLEATE CELLS IN RPL 12 LYMPHOMA
INFECTED WITH ST. LOUIS ENCEPHALITIS

Percentage of tumor cells in	Uninfected	Virus infected	Difference between means	Standard error of difference between means
Prophase	1.84	1.63	-0.21	0.19
Metaphase	2.51	4.88	+2.37	0.43
Anaphase	0.51	0.47	-0.04	0.13
Telophase	0.64	0.38	-0.26	0.13
Mitosis (total)	5.50	7.36	+1.86	1.44
Binucleate cells (per cent of tumor cells)	0.11	1.15	+1.04	0.19

ence of this lipid mass may interfere mechanically with the separation of chromosomes at the end of metaphase.

Discussion

Considerable evidence has been accumulated that the formation of lipid globules is a common feature of infected and uninfected degenerating cells. Alteration in the ribonucleoprotein metabolism of the nucleus in Bunyamwera virus infection is suggested by the frequent formation of numerous intranuclear RNA protein inclusions. Similar RNA protein inclusions are found much less

commonly in uninfected cells, in the rat HeLa cells, they appear to be the result of ischemia. The great increase in the number of inclusions during virus infection may be due to disorganization of nuclear RNA metabolism associated with synthesis of virus. In cells infected with Bunyamwera virus no alteration in the Golgi lipid can be found and the inclusions remain intranuclear until the nucleus disintegrates by karyorrhexis. In Coxsackie B3 and NDV infection no intranuclear inclusions are formed, but there is a conspicuous alteration and increase in the Golgi lipid. There is evidence that nuclear RNA-protein is extruded into the abnormal Golgi lipid where, at least in the case of NDV, it may be surrounded by phospholipid to form cytoplasmic inclusions. RNA-protein of virus origin cannot be distinguished by present cytochemical methods from that of the cell. In view of the demonstrable infectivity of the RNA from cells infected with a number of different viruses,^{2, 29} it is possible that the RNA protein extruded from the nucleus may be concerned with the synthesis of the infectious particle. Indeed, this type of developmental cycle has been postulated by Tournier *et al*³⁰ for varicella virus. Simple particles are formed in the nucleus, from which they pass to the cytoplasm acquiring increasing morphologic complexity en route.³⁰ A similar evolution of the infectious particle has been suggested by Fawcett in a study of the Lucke renal carcinoma of the frog.³¹

An increase in the Golgi lipid, with or without the formation of cytoplasmic inclusions, has been found in six of seven virus infections studied. This type of change has not been observed in uninfected tumor cells, but has been described in a number of other virus infections.^{9, 12} The development of juxtannuclear acidophil masses containing basophil granules has been reported in cells infected with poliomyelitis and Coxsackie B3 viruses.^{12, 31} Acidophil cytoplasmic inclusions also have been described by Brandt³² in tissue cultures of chick embryo cells infected with NDV. Although no histochemical studies have been made of these structures, the acidophil inclusions probably correspond to the Golgi phospholipid and the basophilic granules to the RNA protein particles observed in tumor cells infected with Coxsackie B3 and Newcastle disease viruses. Electron microscope studies of monkey kidney cells infected with poliovirus reveal the development of a mass of U, or unknown bodies, in the juxtannuclear zone of the cytoplasm.³² Although Hallman *et al*³³ do not commit themselves as to the nature of the U bodies, they feel fairly confident that these bodies are not virus particles. Since U bodies develop in the Golgi zone, they may be similar to the phospholipid accumulations found in this region in other virus infected cells.

Although changes in the lipid in the Golgi zone are frequently found in virus-infected cells, there is little information to indicate any connection between these lipid changes and viral synthesis and replication. The presence of virus like particles and of antigen in the inclusions of NDV has already been discussed. Howatson and McCulloch³⁴ have described viruslike particles in a large osmophilic inclusion in the Golgi zone of a mouse plasma cell tumor. The particles resemble the viruslike bodies described in mammary tumors of mice.³⁵ These authors have not yet been able to demonstrate that the particles are in fact viruses. The teleological significance of the changes in the Golgi lipid in virus infection is not clear. The hypertrophy and hyperplasia of the

Golgi lipid in cells of the RPL-12 lymphoma in the brain, which are infected with, but not destroyed L. paratus may have a defer lipid bodies described by cell and impede the development of the infectious particle

Summary

Infection of tumor cells with viruses produces changes comparable to those seen in nonneoplastic cells. Certain virus infections produce cytochemically detectable disturbances in the RNA metabolism of the nucleus. In the case of Bunyamwera virus infection, intranuclear ribonucleoprotein condensations or

cles A viruses, in chicken lymphoma cells infected with St. Louis encephalitis virus, and in rat HeLa cells infected with Coxsackie B3 virus. Many chicken lymphoma cells infected with St. Louis encephalitis remain viable, but the abnormal accumulation of lipid in the Golgi zone appears to interfere with mitotic division. Circumscribed structureless foci containing protein but no nucleic acid are found in the cytoplasm of Ehrlich ascites tumor cells infected with Anopheles A virus.

Intranuclear RNA protein inclusions, like those in virus infected tumor cell have been found much less frequently in uninfected degenerating cells. Although this abnormality of nuclear RNA protein can be produced by virus infection it may also result from ischemia and other causes.

Acknowledgments

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CELLULAR RESPONSES TO INFECTION WITH STRAINS OF HERPES SIMPLEX VIRUS

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Introduction

The characteristics of herpes infected tissues, both animal and egg have been well described. In general, the tissues respond by ballooning of cells and the formation of multinucleated giant cells, the nuclei of the infected cells being altered by the formation of inclusion bodies. Scherer¹ described the appearance of intranuclear inclusion bodies in strain L mouse fibroblasts, and later Scherer and Syvertsen² pointed out that infected HeLa cells became rounded and gathered in small clumps but in addition, they described a few foci of degeneration. Stulberg and Schapira³ described the ability of herpes virus to grow on fibroblasts derived from various tissues of eleven-day-old chick embryos. They reported the appearance of focal areas of necrosis with cells containing intranuclear inclusions at the margins of these areas.

of syncytial giant cells and also by the piling up of rounded ballooned cells. They utilized the technique of terminal dilutions to isolate different populations

apparent piling up. They called the first virus population giant cell or GC strain, the second proliferative or P strain, and the third nonproliferative or NP strain.

Development of Strains

Further observations have suggested that a true difference exists between the virus strain that produces giant cells and that which does not, and that the exact picture of the latter may depend on circumstances such as the char-

cells of either type resemble bunches of grapes on the glass. In this paper the strains will be designated as GC for syncytial giant cell producing virus and P for nongiant cell producing virus.

More precise separation of the P and GC strains has been achieved by utilizing the technique of Kaplan⁴. Herpes virus is grown on rabbit kidney tissue culture under agar and isolated plaques are picked for subculture. By this means two strains GCA₂ and PA₂ have been isolated and studied and most of the responses demonstrated in this paper have been produced by these two subcultures. TABLE 1 shows the source of these two strains and the labora-

tory manipulations that they have undergone human clinical lesions

It should be noted in passing that when virus has been isolated from human lesions, whether primary or recurrent, and in tissue culture whether HeLa, human amnion, or rabbit kidney, careful observation of the original tubes has

Both strains were derived from

TABLE I
SOURCE OF STRAINS

GCA ₁	PA ₁
From recurrent vesicle, May 23, 1955	From vulval vesicle March 7, 1957
<div> CAM </div> <div> HeLa = mixed May 25, 1955 </div> <div> Frozen at -70° C </div> <div> HeLa April 29, 1957 </div> <div> HeLa (45) </div> <div> Line amnion under immunosuppression Jan 20, 1958 </div> <div> Plaque </div> <div> Line amnion (5) </div> <div> Rabbit kidney, Feb 20, 1958 </div> <div> Rabbit kidney under agar, (10) May 29, 1958 </div> <div> Plaque = GCA₁ </div> <div> Rabbit kidney (6) </div> <div> Stock virus at -70° C </div>	<div> HeLa = chiefly P </div> <div> HeLa (19) kept at -70° C in intervals </div> <div> Rabbit kidney under agar May 27, 1958 </div> <div> Plaque = PA₁ </div> <div> Rabbit kidney (4) </div> <div> Stock virus at -70° C </div>

Numbers in parentheses indicate number of passages

usually revealed evidence of both types of response. In our experience it is more usual to find primarily a proliferative response with few if any giant cells. Occasionally giant cells seem to predominate.

Giant Cell Strain (GC)

The histological changes seen in the giant cell strain are as follows. In an ill-defined focus of infection among the normal cells may be seen a group of

scattered nuclei, which on close inspection seem to be those of swollen cells, the boundaries of which are contiguous or absent as the intervening membranes apparently lyse. Normal-appearing cells may be seen at the margin as if about to be incorporated. Later, the area of fusion enlarges and the nuclei appear to be crowded together. Still later there appears to be shrinkage which is marked in the middle of the area. In the final stage of fusion, the nuclei are crowded together so closely that most of the nuclei are lost, although



FIGURE 1 Early formation of syncytial giant cell. The nuclei are seen lying separated in cytoplasm; the boundaries of the cells sometimes may be distinguished. Rabbit kidney, Giemsa stain, tube. $\times 75$.

some could not be distinguished readily from normal cells (FIGURES 1 to 5). Occasionally individual nuclei are very large, and sometimes, in a cell, one or several nuclei, each containing an inclusion body, appear as if they may have just divided.

P Strain

The cells appear on top of the monolayer as a small, rounded, piled up area of single cells. As the virus passes, the surrounding cells of the monolayer seem to become attached to the pile, so that the focus lies as if shrunk away from the monolayer and finally only piles of cells, like bunches of grapes, may be seen attached to the glass. The rounded cells, form-



FIGURE 2. More advanced lesions with several masses of nucleating empty areas in which no cell boundaries may be seen. Rabbit kidney. Gomori stain. tube. $\times 68$.

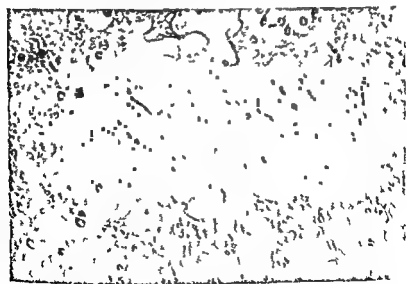


FIGURE 3. Syncytial formation similar to FIGURE 2 but in HeLa cells. Living culture. HeLa tube. $\times 68$.

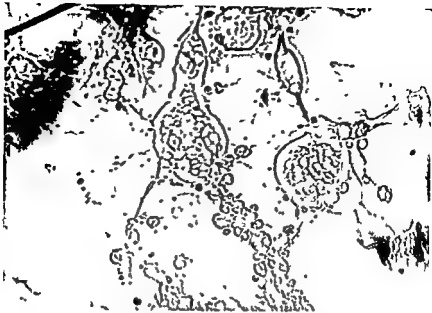


FIGURE 4 Late appearance of giant cells in which they are rounded and apparently contracted with central masses of nuclei surrounded by cytoplasm. Living culture rat let kidney tube $\times 68$

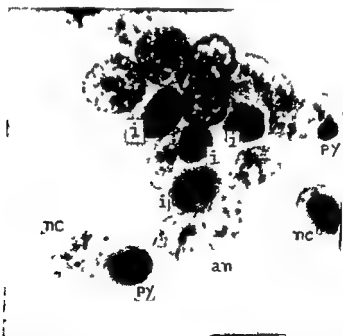


FIGURE 5 Intermediate stage syncytial giant cell showing appearance of intranuclear inclusions of various stages, as well as some nuclei that are altered little *an*. Two dead cells with pyknotic nuclei *py* are present and two normal cells *nc* in contact with the edge of the giant cell. HeLa II and E. sta. n. tube $\times 300$

ing the pocklike focus, chiefly show evidence of infection by the presence of inclusion bodies. Some of the cells at the edge may have normal appearing nuclei but in a field of such thickness it is difficult to distinguish nuclear details (FIGURES 6 to 10).

The host cells that respond with proliferation not infrequently also produce a small number of small giant cells, but the syncytial masses are never seen. This striking difference can be demonstrated in comparing very advanced states of each strain on primary human amnion cells (FIGURES 11 and 12).

Effect on Pure Fibroblasts

In addition to producing the effect of these two strains on HeLa amnion and rabbit kidney tissue it was also possible to infect chick embryo fibroblasts derived from eleven-day-old chick embryos with both strains. In this tissue it was easy to find giant cells produced by the GC strain. The pocks of the P strain were harder to recognize because of the irregular nature of the monolayer, but we never saw syncytial formation with the P strain and thought that we could recognize proliferative foci in the stained if not in the living tissue.

Other Characteristics

It was obviously of interest to determine if these two populations of virus particles had other differentiating characteristics that might suggest genetic markers. The information available is summarized in TABLE 2 and is very disappointing from that point of view. Titration in tubes indicates that both strains grow almost equally in the various hosts tested. Although the data presented show a tendency for the GCA₂ strain to have a higher titer than the PA₂ strain, this difference is not consistent. The GC₂ plaques formed on the rabbit kidney cells do appear to be somewhat larger 4 to 5 mm in diameter than the PA₂ plaques, 2 to 3 mm in diameter however there is sufficient variation in each to make consistent recognition difficult. (In more recent studies with controlled pH, there appears to be no difference in the rate of heat degradation at 37° C of the two strains, although we had thought from earlier experiments that the GC strain was less sensitive. Ultraviolet light appears to inactivate both strains at the same rate. Both strains adsorb onto rabbit kidney cells at the same rate, 75 and 80 per cent of the GCA₂ and PA₂ respectively being adsorbed in 2 hours whereas newly formed virus in each instance appeared in the medium after 7 hours. Electron microscope pictures thus far have not revealed any consistent difference between the sizes of the virus particles derived from the two strains. Each strain has retained its pathogenicity for animals, there is no difference in titer in weanling mice and rabbits respond to corneal scarification by the development of a keratoconjunctivitis without encephalitis. The response of the chorioallantoic membrane of the embryonated hen's egg (CAM) deserves some comment. It is interesting that both strains produce typical pocks, but the difference is obviously insufficient to be a useful marker. Passage of each strain in CAM for as many as eight passages did not appear to alter its capacity to produce a specific response in tissue culture; the passaged P strain produced piled up pocks and the passaged GC strain syncytial giant cells.

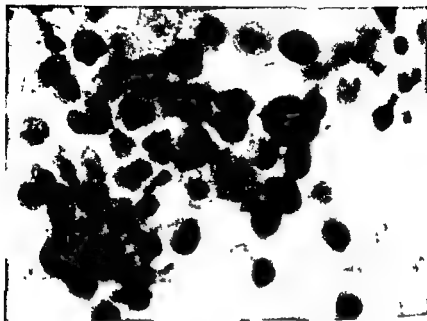


FIGURE 10 Edge of pox, showing intranuclear inclusion bodies in most of the cells in focus at the top of the pile. HeLa, H and I stain tube X270



FIGURE 11 Late giant cell formation (GC strain) Primary human amnion, Giemsa stain, tube X68



Fig. 12 Late pox formation (P strain) in Giemsa stain tube X64 Note small giant cell (C) P n r

TABLE 2
COMPARISON OF STRAINS

Hosts	GC A	PA
Rh cells ID ₅₀ /ml	10 ^{-4.4}	10 ⁻⁴
Rh cells PFU/ml	1.4 × 10 ⁴ la ger?	1.5 × 10 ⁴ smaller
Hel a cells ID ₅₀ /ml	10 ^{-4.4}	10 ⁻⁴
Chick embryo fibroblast cells	2 × 10 ⁴ larger?	2 × 10 ⁴ smaller?
CAV PFU/ml	10 ^{-4.4}	10 ⁻⁴
Heating in ice LD ₅₀ /ml	10 ^{-4.4}	10 ⁻⁴
Label its cornea	+	+
keratoconjunctivitis	2 2 3 3 hours	2 0-3 5 hours
encephalitis	9-12 m n	9 12 m n
Heat degradation at 37° C 1/2 life		
Ultraviolet inactivation at 8 m distance		

Summary

In summary it would appear that it is possible to derive from freshly isolated human herpes virus at least two types of infecting agent one of which leads to the breakdown of the boundaries of the host cells in the process of infection with the formation of syncytial giant cells the other appears to produce a piling up and apparent proliferation of the host cells without any tendency for cell boundaries to lyse. Since these responses can be produced at will it may

TABLE 1
MULTIPLICATION OF ISOLATED HFLA CELLS IN MICRODROPS
FOLLOWING EXPOSURE TO HERPES VIRUS

Multiplicity of infection HFLA PFU per cell	24 hours		72 hours		Observed proportion of cells with inhibition of mitosis*	Theoretical proportion on infected with one or more infectous units
	Total examined	Proportion divided	Total examined	Proportion divided		
uninfected	181	0.46	181	0.76	—	—
0.1	90	0.28	70	0.68	0.10	0.09
1.0	120	0.12	40	0.27	0.64	0.63
2.7	40	0.05	40	0.12	0.84	0.93

* Proportion of cells in infected cultures dividing by 72 hours

Proportion of cells in uninfected cultures dividing by 72 hours

Virus treated at 50° C for 1 hour had no antimitotic activity, and the antimitotic activity was reduced by addition of herpes anti-serum. Additional normal serum also had a slight protective effect on the cells, but the difference was significantly less than that produced by anti-serum.

Assuming that 76 per cent of the cells would have divided by 72 hours if they had not been exposed to virus, it is possible to calculate the proportion in which division was inhibited. This is found to agree closely with the theoretical number of cells adsorbing one or more HFLA PFU as deduced from the Poisson distribution. We can conclude that one HFLA PFU is sufficient to inhibit mitosis, because about half the cells affected should not have absorbed more than one PFU per cell.

It is known from other experiments that there are about ten times as many chick cell infective particles as HFLA infective particles in the seed suspension, and it is also known that the chick cell infective particles adsorb to the HFLA cells, although the majority do not initiate infection (Stoker and Ross, 1958). In the experiments with single HFLA cells described above, a multiplicity of 0.1 HFLA PFU per cell would probably be accompanied by adsorption of about 1.0 chick cell infective unit per cell. It is obvious from the high proportion of dividing cells that this does not inhibit mitosis.

With the higher doses of virus the proportion of cells releasing infective virus into the microdrops was small and not related to input. This failure of virus release from cells which nevertheless initiate plaques (infective centers) in uninfected HFLA cells is discussed elsewhere (Stoker, 1959), but it should be noted that none of the cells yielding virus divided, none formed multinucleate giants; the majority were rounded, and some had already disappeared when the sample containing virus was taken.

Kinetics of Mitotic Inhibition

Since mitotic inhibition is a function of the time of exposure to virus, we investigated the kinetics of mitotic inhibition. This was done by exposing cells to virus (parasynchronously),

When exponentially growing monolayers of HFLA cells at 37° C are chilled to 4° C for one hour, then returned to 37° C, the cell numbers remain almost

stationary for 18 hours. The majority of cells then divide within 1 to 2 hours. This is followed by a stationary phase of 18 hours and then a second increase in cell numbers lasting 1 to 2 hours. Thereafter the cultures become too crowded for exponential division. The proportion of normal cells dividing usually varies from 60 to 80 per cent. Those cells that do not divide parasynchronously never divide, and so they do not confuse the step-like pattern of growth. Full details of the procedure are given by Newton and Wildy (1959).

To investigate the time between virus attachment and inhibition of mitosis, replicate cultures of HeLa cells were chilled and at different time intervals during the first stationary phase were exposed to herpes virus suspension for an adsorption period of one hour, after which herpes anti-serum was added to prevent further infection. The dose of virus was sufficient to infect about two thirds of the cells. Parallel cultures were inoculated with medium containing no virus. Cell counts were made at intervals on sets of four or more replicate cultures.

The uninfected cultures and cultures exposed to virus and anti-serum divided at 18 hours and again at about 36 hours, while the majority of cells in the infected cultures failed to divide at all. The inhibition of cell division took place even when the virus was first added $16\frac{1}{2}$ hours after chilling 90 min. before expected division. Since neither infection nor cell division are quite synchronous, it is not possible to estimate accurately from this the time interval that elapses between virus adsorption and the damage to mitosis. Adsorption is rather slow, however, and 1 hour must elapse before sufficient virus attaches to infect two thirds of the cells. This would have been complete $17\frac{1}{2}$ hours after chilling, or 30 min. before cell division should have commenced. It seems probable that the damage to mitosis occurs within 60 min. of virus attachment and, of course, it may be much less. This may be compared to a lag phase of 9 to 12 hours before new virus appears in the cell fraction, and it suggests that as in certain types of bacteriophage, damage to mitosis is a relatively early event in the cycle of virus replication. It is of some interest that this inter-

of them retain certain of the characteristics of living cells. They remain rounded, although morphologically intact. They stain with neutral red and not with trypan blue. Production of acid suggests that in some respects they are more active metabolically than uninfected cells. In intact monolayer cul-

this need not apply to other strains of herpes virus or other cells. For example,

factory evidence for this process has been found only with Rous sarcoma virus in cultured chick cells (H. Temin and H. Rubin, unpublished data).

The tissue changes induced by several viruses seem to suggest proliferation of infected cells, but it is not possible to define accurately the relationship between virus growth and cell division without quantitative studies *in vitro*. Although our investigations showed no evidence of continued division of the infected cell, the techniques involved may be applicable to the investigation of other virus-cell systems.

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ELECTRON MICROSCOPE STUDIES OF CELLS INFECTED WITH THE SALIVARY GLAND VIRUSES*

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Electron microscopy has stimulated morphologic study of viral diseases in particular, since visualization of the infective agent is now possible. Particles associated with many virus diseases have already been identified and work has begun in the complex area of viral life cycles. The mechanisms whereby viruses gain access to the interior of cells, the fate of virus particles after penetration of the cell, and the site and type of morphologic changes accompanying viral growth all need to be examined. With these problems in mind we shall present here not only the alterations occurring in distinctly diseased cells, but also early changes evident after the addition of human salivary gland virus to tissue cultures of human fibroblasts.

Materials and Methods

Cells were maintained in a medium composed of 85 per cent beef amniotic fluid, 10 per cent beef embryo extract, and 5 per cent horse serum. All media contained 100 U of penicillin and 100 μ g of streptomycin per ml. Four series of experiments were carried out. In 2 of these, pooled media from cultures infected 10 days previously were filtered through a 0.15 Celas filter, and an inoculum of 0.5 ml of the filtrate was added to 1.5 ml of fresh media for each roller tube. In the other 2 series, media from infected cultures were centrifuged and 0.5 ml of supernatant added to the fresh media of each tube. Cultures were fixed at 2-day intervals following inoculation. Parallel control cultures were obtained at 2 to 3-day intervals. Immediate control cultures received an inoculum of supernatant from uninfected cultures equal in amount to the infected supernatant added to experimental cultures.

Both normal and infected cells were prepared for electron microscopy by fixation in Dalton's osmic acid fixative (1 per cent osmium tetroxide in dichromate) for 10 to 15 min.² Cells were fixed and dehydrated *in situ* in the roller tube after decanting the culture media. Dehydration was by graded solutions of ethanol (10 to 100 per cent) over a total period of 1 hour. The cell colonies were removed from the tube with a dental elevator and placed in one half absolute ethanol and one half methacrylate. They were infiltrated in 3 changes of 7:1 butyl methyl methacrylate for a total of 1 hour. A catalyst benzoyl peroxide, was added to the last change of the plastic. The fibroblasts were embedded in gelatin capsules filled with the slightly polymerized methacrylate mixture. Polymerization was at 60° C for 12 hours. Blocks were sectioned

Original electronmicrographs were made at 2000 to 12,000 diameters on Cramer contrast plates, developed in Kodak D-61 diluted one half with water and subsequently enlarged photographically as desired

Results

Control Normal fibroblasts in tissue culture were elongated cells with rather scant cytoplasm that often extended into attenuated prolongations (FIGURE 1). The nuclei were ovoid, although occasionally indented with one or more prominent nucleoli. The nuclear chromatin was finely granular and



FIGURE 1. Electron micrograph of normal elongated fibroblast in tissue culture. Part of the nucleus is evident at the left. The cytoplasm contains elongated narrow ergastoplasmic sacs. Mitochondria are scant and small. At approximately $\times 6000$.

uniformly distributed with a distinct increase in density near the nuclear membrane. Mitotic figures were rare. The cytoplasmic ergastoplasm was arranged as parallel rows of membranous envelopes with more or less prominent dense granules present along their outer margins. The inconspicuous Golgi material was evident as clusters of small vesicles in the perinuclear region. Mitochondria were sparse and elongated with distinct cristae. Occasional intracytoplasmic inclusions of lipid occurred. A zone of fine intracytoplasmic tonofibrils was present at the inner margin of the plasma membrane of some fibroblasts. Normal fibroblasts occasionally had moderately abundant cytoplasm rather than being spindle shaped. Cells of control cultures to which supernate from noninfected cultures had been added a few minutes prior to fixation showed some dilatation of ergastoplasmic sacs and a questionable increase in cytoplasmic vesicles in comparison to cells of cultures of the same age incubated for 2 or 3 days without change of the media. A majority of the cells remained

spindle shaped and the changes were minimal in comparison with those of cells exposed for a longer time.

Cells after inoculation were similar in both the cultures examined immediately and in those examined 5 min after inoculation, consisted of a rounding up of cells with an apparent increase in



FIGURE 2. Electron micrograph of a cell.

virus
is plump
cytoplasm

cytoplasmic volume. There were numerous pinocytosis vacuoles evident (FIGURE 2), some of these contained electron dense material. The ergastoplasmic sacs were frequently dilated and filled with an opaque homogeneous material. There was a multitude of minute vesicles within the cytoplasm (FIGURES 2 and 3). Mitochondria were unaltered. For the most part the nuclei were unaltered, but an irregular rim of dense material was present at the nuclear margin in some cells.

Viral particles were not identified at the outer margins of cells or between

abnormal although the nuclei remained relatively unchanged. The cytoplasm was increased in amount and many cells were almost spherical (FIGURE 8). Small mitochondria were numerous. The ergastoplasmic sacs of some cells



showed

ed

..

were distended in others they were inconspicuous the cytoplasm being filled with numerous small vesicles. The Golgi substance was prominent.

The earliest definitely abnormal nuclei with nuclear inclusions were identified 2 to 3 days after inoculation. At this time the central portion of the nucleus was partially filled with an inclusion containing particles composed of a small dense central body surrounded by a pale halo and an outer dense membrane.

(FIGURE 9) The particles in these inclusions measured approximately 60 to 90 $m\mu$ in diameter. They were interspersed within a more dense granular material and the entire inclusion was surrounded by a pale region between it and the nuclear membrane that was distinctly increased in density. During development of a nuclear inclusion the number and density of particles increased (FIGURE 10). Nucleoli remained unaltered or became somewhat more



..

prominent. No stage of altered nuclear morphology preceding the appearance of viral particles was identified definitely. At the time of the appearance of particles and of the nuclear inclusion, identifiable virus particles were not evident in the cytoplasm.

pl
ou
viral particles have been described previously. Two types of cytoplasmic



FIGURE 12. Electron micrograph of a cell showing a nucleus (NP) and a large, irregularly shaped cell.

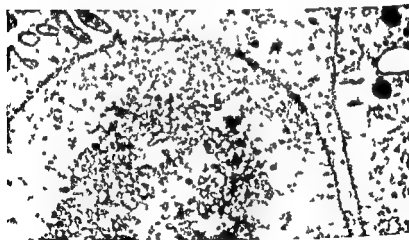


FIGURE 13.

particles associated with infected cells were recognized (FIGURE 13). One averaging about 80 to 100 $m\mu$ in diameter was a target form comprised of a central dense dot surrounded by a single or double rim of dense homogeneous material. The other type of particle consisted of a solid sphere varying from 150 to over 500 $m\mu$ in diameter. Both types of particles were within membranes some of

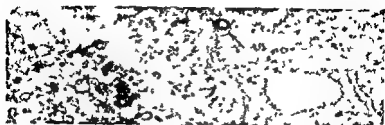


FIGURE 11 The double nuclear membrane is separated at the upper right arrow and encloses a dense particle that is similar to the mature type of particle seen in the cytoplasm (compare with FIGURE 13). $\times 25,000$

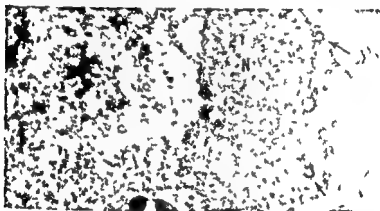


FIGURE 12 A section through part of the nucleus of another fibroblast infected 10 days previously. The nucleolus is apparently unaltered. At the arrows 2 particles similar to the one seen in FIGURE 11 are present between the two membranes surrounding the nucleus. $\times 20,000$

which appeared to be those of the ergastoplasm. In the older cultures numerous free particles were noted between cells.

Discussion

penetration of the cell since the latter occurs with such rapidity. From these studies it appears that the process by which virus particles gain access to the cell is not one of direct combination of the particle with the cell surface but rather one of engulfment. Indeed rather large masses of material are evident within the cytoplasm of some fibroblasts (FIGURE 7). Following addition of media from cultures of uninfected fibroblasts control cultures show only a minimal increase in pinocytosis or dilatation of ergastoplasmic sacs. Rapid and

extension pinocytosis may be due to addition of protein which stimulates the process.⁸ We have not yet examined cultures following addition of heat killed virus, and do not know whether addition of killed viral suspensions would lead to a response similar to that containing viable virus. The rapidity of pinocytosis frequently observed in living tissue culture cells is neither surprising nor unusual.⁹

The fate of virus particles during and after entrance into the cell remains unknown. Occasionally particles reminiscent of target forms of the virus are seen within cells recently exposed to virus. Whether these forms are identical with those in the inoculum is not known. The definitive changes in virus particles during penetration of the host cell are not yet understood. The virus

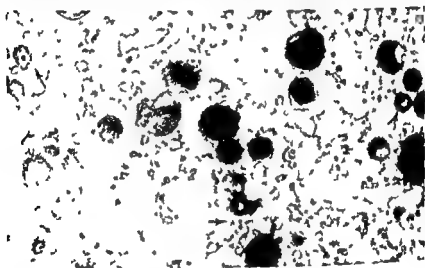


FIGURE 4

may well disappear secondary to a structural alteration of protein molecules after or during penetration of the cell membrane.

The late alterations of cells infected by salivary gland virus have been described by both light^{7, 8} and electron⁴ microscopy. The early changes prior to the classic cytomegalic picture, however, have not been described in any detail previously. As we have shown the fibroblast infected 10 to 20 days previously is a large cell containing both nuclear and cytoplasmic viral particles. In light of this it is most interesting to see early cytoplasmic changes with ingestion of particulate material and enlargement and rounding up of the cell but with no early nuclear alteration. Two to 3 days later, a nuclear inclusion of viral particles has formed while none is evident in the cytoplasm until about the fifth day after infection. This raises the question of the manner in which infective material reaches the nucleus, as well as the question of our ability to recognize early pathological alteration of chromatin.

The nucleus certainly is not isolated from the cytoplasm. Watson and others have clearly shown that the ergastoplasmic sacs are continuous with the outer nuclear membrane as well as with the plasma membrane. We have seen mature virus particles between the two nuclear membranes in uninfected cells² (FIGURES 12 and 13). That a virus may leave the nucleus to reach the cytoplasm is, however, a different matter than for the

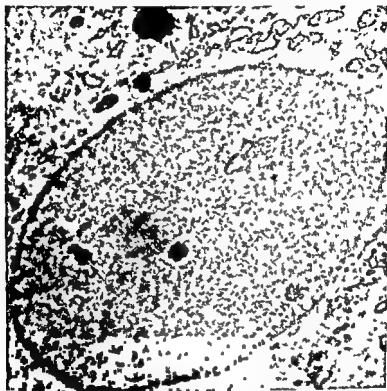


FIGURE 14. Electron micrograph of the nucleus and part of the cytoplasm of a rabbit yolk sac epithelial cell. Twenty-four hours previously India ink had been injected into the uterine lumen. Ink (arrows) is present in both the nucleus and cytoplasm. $\times 12,000$.

intact nucleus by extracellular or intracytoplasmic foreign particles. There is definite evidence that the latter does occur. Following injection of a suspension of colloidal carbon (India ink) into the uterine lumen of the pregnant rabbit yolk sac, the ink is found in the cytoplasm and nucleus of the yolk sac epithelial cells. Whether these materials entered the nucleus by means of the well-demonstrated nuclear pores¹⁰ or by a mechanism akin to pinocytosis¹¹ by the nuclear membrane is not clear at this time.

basal layer with the inclusion bodies becoming increasingly evident and maturing toward the surface

100 μ 50 μ 25 μ 10 μ

The nucleus is pushed to one side and the nuclear membrane is markedly thickened. The nucleus contains comparatively large dense granules sparsely scattered throughout the nucleoplasm which distinguishes it from the nucleus of the uninfected cell. Mitochondria with their characteristic cristae are seen at the periphery of the cell. Near the nucleus are several other bodies that do not have cristae but seem to be associated with the viral infection. They are sur-



FIGURE 3. A cell of molluscan contagium surrounded by epidermis which appears enlarged. Inclusions appear in the cytoplasm covered by a mass of molluscum bodies.

rounded by a membrane and have a granular content. There is a thin rim of cytoplasm separating nucleus and inclusion body from the cell membrane and within the cytoplasm a few strands of keratin are present. The inclusion body contains locules of elementary bodies separated by septa of complex composition in which varying forms of the developing elementary bodies are present. The entire cell is surrounded by a convoluted membrane. This is the general picture of an infected cell. How this picture evolved, how it progresses and the finer details of the various structures mentioned are of considerable interest.

Nucleus. In an infected cell the nucleus and nucleolus are usually enlarged but as the inclusion grows the nucleus apparently shrinks and large folds appear in the nuclear membrane (FIGURE 3). The nucleus is pushed to one side, becomes flattened and on sectioning appears elongated. Unique to the nucleus of the infected cell are relatively large dark bodies of varying size and of slightly

irregular shape that are present in a variable number and in the nucleoplasm.

Cytopla — The organelles of the infected cell are on the surface of the nucleus and are developed by the developing nucleoplasmal homomorphous elements.



FIGURE 2. Cell of a mollusc infected by a molluscum contagiosum virus. The nucleus is on the left and the cytoplasm is on the right. The granules in the cytoplasm are the developing nucleoplasmal homomorphous elements. $\times 300$.

rapped. The mitochondria are displayed and occasionally are lumped (Figs 4 and 5).

Bodies of the order of 22 to 900 μ are of evidence in the cytoplasm about the nucleus or even at a distance from it and are designated as C for cytopla-

bodies (FIGURES 6 and 7). The larger ones often have a distinctly double membrane measuring $\approx 165 \text{ \AA}$ in thickness with the space between the two unit

stances the membrane appears to have overlapped itself since 3 dense unit membranes are seen. The contents of these structures are usually sparse and composed of reticular and granular material, although relatively large bodies or an occasional vesicle may be present within them. The double membrane

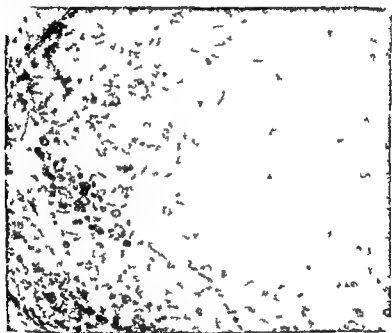


Electron micrograph

surrounding them appears coarser than the membranes of the mitochondria present in the same photograph, and the unit membranes themselves are wider and darker than most of the mitochondrial unit membranes. When contrasted with the nuclear membrane, it is darker and does not have a surrounding condensed rim of cytoplasm.

Developmental structure of inclusion body. In the earlier stages of infection there appears in the cytoplasm of the cell material similar to that making up the septa of the more mature inclusion body. This early inclusion pushes back the mitochondria and the nucleus (FIGURES 2 and 4). In this respect it seems to replace the cytoplasm, but it does not do so entirely, for at least one component, the keratin fibrils, is often entrapped (FIGURES 5 and 8).

The material composing the early inclusion contains two types of ground structures: one is finely reticular although it may appear amorphous at low magnifications and the other granular occurring in localized areas (FIGURES 2, 9 and 10). The reticular fibrils are very fine, some at the limit of resolution of the microscope of electron with tiny dense nodes at the points of intersection (FIGURE 10). The granules of the granular component are in the order of 130 to 160 Å in diameter and are apparently embedded in a condensed substructure (FIGURE 9). That is to say, tiny strands can be seen running between the



granule or they may be indistinct because of an amorphous material surrounding them.

When the ground substance of the early inclusion other structures are present (FIGURE 11). One is a round or oval profile ranging from 640 to over 3000 Å. They are surrounded by a membrane which is either clear to moderately dense. The contents are either homogeneous or contain a dense bar or there is a suggestion of a few granules. The second type of profile is that of a dense granule in the order of 250 to 850 Å in diameter surrounded by the smaller granules of the granular ground substance. All these structures generally occur in foci and are associated with the granular ground substance.

The above structures are clearly part of the early inclusion. There are



FIGURE 5



FIGURE 6 C bodies (C and brackets) in cytoplasm. Portion of nucleus at bottom
X7400

others however that probably appear later and are seen regularly in the septa of older molluscum bodies. These are of four types and do not seem to be

a crudely elliptical profile in the order of 90 μ in diameter it is separated



FIGURE 7. C body. Note granular and reticular inner structure, double membrane, and the fibrillar nature of the tangentially cut membrane. $\times 36,000$.

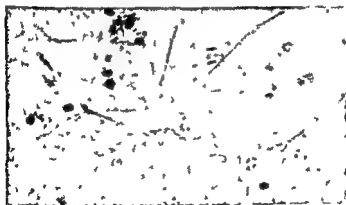


FIGURE 8. Nucleoid. Note dense nucleoid surrounded by a clear zone, indicating a clear zone between it and the surrounding membrane. $\times 36,000$.

from the rest of the contents by a less dense zone (FIGURE 12b). The nucleoid is often much denser than the rest of the contents, which are also reticular. The size of the nucleoid varies, although some of the variation in size and density may be the result of tangential sectioning. It is often present in a larger structure than Type II and at times the nucleoid contains a dense central portion on which condensed, leaving a clear zone between it and the surrounding membrane (FIGURE 13).



FIGURE 9. Reticular ground substance and focal areas of granular ground substance of the molluscum body. Type II and Type IV developmental forms of the elementary body are present in the lower right. Some forms of the mature elementary body are also present. $\times 44,000$.

Type II is a round compact body with or without a membrane (FIGURE 14). The membrane which is present in some cases and appears single making up the body. In such a structure a denser eccentric area arising from the membrane. In such a structure a denser eccentric area

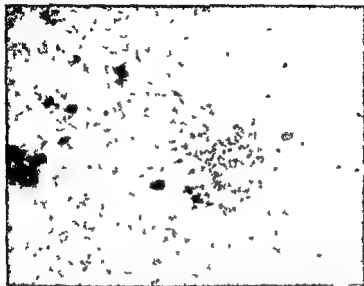


FIGURE 10. Reticular and granular ground substance. Note nodal thickenings at points of intersection of fine filaments of the reticular ground substance. Granular ground substance is clumped. Large dark bodies are portions of elementary bodies. The electron filaments are present. Fixation and staining as in FIGURE 8. $\times 17,000$.

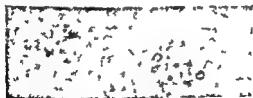


FIGURE 11. Reticular ground substance surrounding foci of granular ground substance. These foci contain round and oval profiles and relatively large dense granules with small granules clustered about them. $\times 19,000$.

suggestive of a disappearing nucleoid sometimes seen in the central dense plasma (FIGURE 15).

Type III is a lobulated structure of a reticular moderately dense material with narrow spaces.

Type IV is a dense structure to be seen least frequently.

membranes are separated by homogeneous moderately dense wall material that increases in thickness as the central areas beneath the broad convex surfaces of the elementary body are approached. In the rectangular plane



FIGURE 13 Type I nucleoid containing structure (arrow). Other developmental forms are present in the septa and mature elementary bodies in the locules. $\times 21,000$



FIGURE 14 Type II round to oval profiles with dense interiors. Swollen forms of the mature elementary body are present. $\times 20,000$

however, the two membranes often appear to meet at the margins. FIGURE 22 is a drawing of the proposed basic structure of the elementary body.

Within the mature elementary body is a dense granule (FIGURES 18 to 21)

It is from 50 to 100 Å in

width It is often

surrounded by tape. Most

frequently it occurs associated with the thickened portion of the wall but may be seen on the inner membrane or within the less dense central area.

Its occurrence in the mature elementary body is such that when the thickness of the section is considered together with the dimensions of the elementary body it would appear that there is one dense granule for each elementary body.

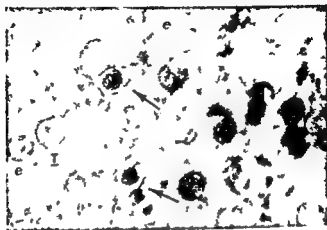


FIGURE 15
Elementary

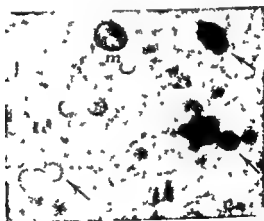


FIGURE 16 Type III germinating forms (arrows) M mitochondrion $\times 19,000$

This dense granule also occurs in the extended forms of the virus usually associated with the thickened portion of the wall; however, they may be seen occasionally within the less dense central area. It has been seen in Types II and IV of the developing elementary body. It has not yet been seen in Types I or III and does not seem to appear outside the elementary body. Occasionally two bodies, both of which could be interpreted as the dense granule, are seen in the mature condensed elementary body or in the developmental forms.



FIGURE 17 Type III, in cytoplasm at top of figure. Lobulated form, with some margins covered by membranes. The nucleus at the bottom of the figure contains large dark granules ($\times 15,000$)

This granule does not seem to be unique for molluscum contagiosum, since it has been seen also by us in sections of the Shope fibroma elementary body, and seems to be present in Figures 6, 8, and 9 of Bernhard *et al.*,² which show elementary bodies of the Shope fibroma virus. It has not been seen in vaccinia



FIGURE 18 Swollen forms and condensed forms of the mature elementary body. A portion of septa is seen across the bottom. $\times 21,000$



FIGURE 19 Condensed forms of the mature elementary body. Fine fibrils in a reticulated network run between them. Note the small dense granule in the wall of many of the elementary bodies. $\times 22,000$

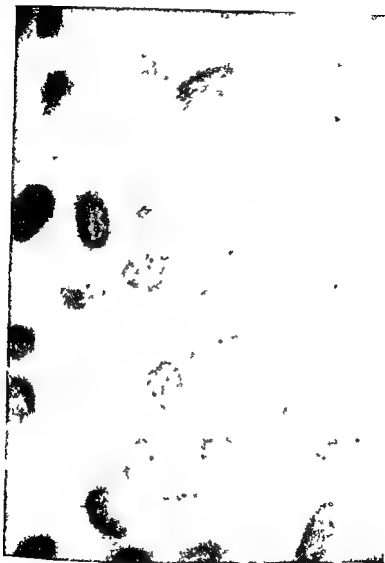


FIGURE 20 Higher magnification of FIGURE 19. Note inner and outer double membranes of the elementary bodies. In some instances a less dense halo appears about the lens granule within the wall. $\times 84,000$

DISCUSSION

Goodpasture and King* have described the extrusion of nucleoli into the cytoplasm and have demonstrated nucleolar-like bodies in the cytoplasm early in the formation of the molluscum body. In the present study relatively large



FIGURE 21 Mature elementary bodies. One section is through the rectangular plane (arrow) $\times 84,000$

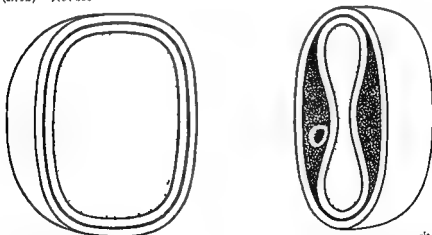


FIGURE 22 Diagram of the proposed structure of the condensed form of the mature elementary body. Section through rectangular plane at left and normal to rectangular plane at right

bodies (FIGURES 6 and 7) were found in the cytoplasm, seemingly associated with early changes in the infected cell. These were the only structures of a size that might appear as extruded nucleoli in the light microscope. They have a distinct membrane that a nucleolus does not have, but this would be

part of the nuclear membrane added as the material passed into the cytoplasm. This membrane, however, is coarser than the nuclear membrane and when the unit membranes are seen, these are denser than those in the nuclear membrane. Their contents at times resemble what might be seen within some nucleoli or within the nucleoplasm. Their membranes are coarser and their contents different from those of mitochondria. Although they were first recognized as large bodies they seem to be related to similar smaller structures (FIGURE 6). Electronmicroscopically, their origin is obscure, and there is no strong evidence that they arise from nucleoli or mitochondria.

The early inclusion is composed of a reticular component and foci of a granular component. It appears that the granular foci give rise to the elementary bodies and therefore would be clearly a viral plasma. The reticular component, however, may be a reaction of the cytoplasm to the presence of the virus.

Van Rooyen¹¹ has shown that the mature elementary bodies are embedded in a jellylike matrix that is seen in the electron microscope as a very sparse reticulum between the elementary bodies. This structure is consistent with that of a gel, and may be derived from the reticular ground substance of the inclusion.

There is a question as to whether the large dense granules within the nucleoplasm are specific for the virus infection or merely a nonspecific result of nuclear degeneration. The work of Dourmashkin *et al.*⁴ would suggest that these granules are specific, since these investigators observed them in HeLa cells infected with the virus of molluscum contagiosum, but not in degenerating cells of the control cultures.

From the present study it is suggested that the elementary bodies of molluscum contagiosum form from the granular foci in the inclusion bodies by the following process (FIGURE 12). Dense granules of the order of 250 Å in diameter appear within the small granular foci. These dense granules increase in size and the small granules organize in a zone about each of the large dense granules. Eventually each dense granule becomes a nucleoid surrounded by a circumscribed reticular plasma with or without a membrane, Type I. After a membrane has developed, the more solid contents including the nucleoid, retract toward the center, leaving an electron optically clear zone between the central dense structure and the membrane. The nucleoid disappears, the membrane contracts, and the remaining particle, Type II, "germinates." The contents grow from the ruptured membrane, possibly fuse with the contents of other "germinating" particles, and grow into a large lobulated mass, Type III. This mass segments into the normal elementary bodies, each of which is

viroplasm differentiates into two symmetrical shells (FIGURE 13). The outer membrane becomes double and an inner double membrane forms about the central area. Thus it is proposed that this may be the sequence by which the mature elementary body is evolved.

The four general types of structure preceding the mature elementary body are never seen within the granular component of the ground substance of the

small dense granule has not been seen in the nucleoid-containing body, Type I the "germinating" structure, or the lobulated structure, Type III.

As noted previously, the mature elementary body may be swollen as the result of the distention of the space within the inner membrane or it may be in a condensed form with the inner membrane collapsed. In section, the swollen forms are slightly elliptical, with the long axis parallel to the thickened portion of the wall. They may reach a diameter of 340 $m\mu$ as against a maximum diameter of 230 $m\mu$ for the condensed forms.

Although the majority of the first formed elementary bodies appear to be swollen and those in the older inclusions near the surface of the lesion to be condensed, this may be the result of immediate environment rather than age, or it may be a combination of both environment and age.

Certainly, Goodpasture and King's suggestion that "the main factor in the hyalinization of the intracellular mass is desiccation" holds true not only for the jellylike material surrounding the elementary bodies, but also for the elementary bodies themselves. The absence of swollen forms in the old inclusions at the surface of the lesion and their abundance toward the base of the lesion would suggest strongly that the swollen forms had shrunk, indicating that at least the outer membrane must be quite elastic.

Summary

The early inclusion body of *Molluscum contagiosum* is composed of two types of ground substance, one is reticular and the other, occurring in focal areas, is granular. This material distends the cell and pushes aside cell organelles. The ground substance of the inclusion continues to increase, particularly the granular component. At the same time, foci of mature elementary bodies are formed apparently at the expense of the granular component. Eventually these foci almost completely fill the cell and divide the ground substance into septa.

Tentatively, this process of elementary body formation begins with the appearance of dense granules of the order of 250 \AA in diameter within the small granular foci. These granules increase in size, and the small granules of the ground substance organize in a zone about each of the larger dense granules. Eventually each dense granule becomes a nucleoid surrounded by a circumscribed reticular plasma with or without a membrane. The nucleoid disappears and the remaining particle "germinates," forming a lobulated mass. This mass segments into the primordial elementary bodies, each of which eventually is surrounded by a single membrane, develops a dense granule, and differentiates into a mature elementary body.

The mature elementary body is found in two forms, one distended and the other condensed. When condensed its shape is that of a rectangular body that appears as an ellipse when sectioned normal to the rectangular plane. An outer and an inner membrane are separated by relatively homogeneous wall

material that increases in thickness as the central area of the
 vertex surfaces of the elementary body are approached. The
 inner membrane surrounds a potential space that is the
 distended form of the elementary body.

Within each mature elementary body is a dense granule
 110 Å. This granule is also present in some of the older
 inclusions in the ground substance of the early inclusion
 bodies. It occurs in the Shope fibroma virus in vaccinia.

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 bodies.

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CYTOPATHOLOGY OF CANINE DISTEMPER STUDIED BY THE USE OF FLUORESCENT ANTIBODIES*

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Canine distemper is a highly infectious disease caused by a filterable virus.^{1,2} It is a common malady of dogs, but it also affects foxes, wolves, ferrets, mink, skunks, and raccoons. The virus has been grown in the chorioallantoic membrane of developing chicken embryos^{3, 4} and is transmissible by intracerebral inoculations to suckling mice and hamsters.^{5, 7}

Canine distemper is of interest not only because of its importance to domestic and fur bearing animals, but also because there is accumulating evidence to show that the virus of this disease may be related in some manner to infection in man. The similarity of certain aspects of the tissue alterations between canine distemper and fatal cases of measles (rubeola) of children has been pointed out, most notably the proliferation of the lining cells of the pulmonary alveoli with the formation of multinucleate giant cells and the presence of both intranuclear and intracytoplasmic eosinophilic virus inclusion bodies.^{8, 9}

A similarity in antigenicity between distemper and a fairly ubiquitous infection conferring immunity in human beings has been suggested because of a rise in quantity from infancy to early childhood of a substance capable of neutralizing distemper virus as titrated in chicken embryos.¹⁰

More recently, experiments conducted by culturing the virus of canine distemper in suckling mice and neutralizing with the measles antisera has shown an immunological relationship between these two diseases.⁶ The transmission of measles to dogs has recently been reported¹¹ and one experiment, at least, has shown that distemper virus inoculated into a man survived for a period of six days.¹² It therefore appears that study of canine distemper is of importance not only because of its value to health of dogs and other animals, but because of its relationship to measles or some other, as yet unidentified, disease of man. Of equal importance, from the standpoint of cytology, is the extreme polyploidism of canine distemper. Because of this, distemper presents itself as does no other disease, as a model for investigation of the effects of such a virus.

Because of slight or aspecific gross changes in canine distemper, supposedly little specific histopathological alteration, and its value in presumptive diagnosis, considerable attention has been centered on the inclusion body. The presence of distinctive bodies in the disease was first reported in the neurons.^{13, 14} Soon their presence in ependymal cells and in bronchiolar and conjunctival epithelium as well as in the neurons, was observed.¹⁵ These inclusions were seen subsequently by many observers and the demonstration of inclusion bodies in tissue sections or smears from various epithelial surfaces came to be used as a diagnostic procedure. Due to this emphasis on the epithelial cell or brain as a site for inclusions and the respiratory or cutaneous nature of many of the signs of the disease, there has been a tendency to ascribe

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primarily epithelial invasive powers to the disease. However evidence to the contrary has been reported by various observers who found characteristic inclusion bodies in lymphocytes, reticuloendothelial cells, endothelial cells, and, probably, other locations¹⁶. In fact, as emphasized below, polytrophism is the most striking characteristic of canine distemper.



FIGURE 1 Photomicrograph of fluorescent antibody preparation of the gastric fundus showing the presence of specific antigenic inclusion bodies in the epithelial cells of the gastric tubuli (f) and within cells of the interstitium (B).



FIGURE 2 Photomicrograph of paraffin section through gastric mucosa at the level of the necks of the glands showing intranuclear inclusion (A), cytoplasmic inclusions in epithelial cells (B), and inclusions in the processes of fibroblasts (C). Hematoxylin and eosin stain.

(6) Lymph nodes and spleen lymphocytes, reticuloendothelial cells leukocytes in abscesses

(7) Central nervous system neurons and their processes, macroglia ependymal cells, and endothelium of blood vessels

In sections studied by means of fluorescent antibody, antigenic material is seen in granules or masses in both the cytoplasm and nucleus of the affected

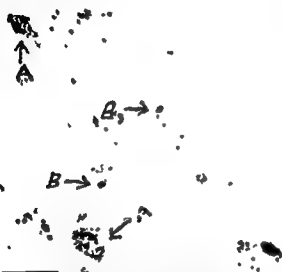


FIGURE 5 Photomicrograph of a fluorescent antibody preparation of the cerebellar peduncle showing specifically fluorescing distemper inclusion bodies in neurons (A) and macroglia (B)



FIGURE 6 Paraffin section of cerebellar peduncle showing hyperplastic vascular endothelium containing cytoplasmic inclusions (canine distemper Hematoxylin and eosin stain)

cells which correspond generally to the size, shape, and distribution of distemper inclusion as seen in paraffin sections. While the material in most locations is nearly always particulate, in the early stage of the invasion it occurs diffusely in the cytoplasm of the reticulum cells and the lymphocytes of the lymphoid tissue. Even in these locations, it later assumes a particulate character. Within the cytoplasm the masses vary in size from minute granules to bodies as large as the nuclei of the affected cells. Intranuclear masses are usually single and of large size.

Most of the body tissues excepting muscle, bone and cartilage may be affected. The specifically fluorescing masses occur even in leukocytes in the



FIGURE 7. Fluorescent antibody preparation of cross section of the neck of a hair follicle showing specifically fluorescent distemper inclusion bodies in the surrounding epithelial cells. (A) Autofluorescence is seen in the hair shaft and in keratotic debris (B and C).

peripheral blood and in phagocytic cells participating primarily in other reactions, for example, foreign body giant cells.

The lesions of canine distemper are rarely diffuse, but seem to occur in disseminated foci in which the inclusions lie in fairly contiguous cells. The affected

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astrocytes, neurons and blood vascular endothelium may be involved in the same focus that often appears related to blood vessels (FIGURE 3).

In ferrets and in a few dogs the virus appears to have severe cytopathogenic

effect, the affected cells being quickly destroyed. In the ferret this may be associated with infiltrations of *neutrophils* and other changes of acute inflammation. Theoretically, it must be

distemper infection in the normal animal is manifested in the dog, not to be accompanied by especially notable in the stomach, where loss of the chief cells with sparing of the parietal cells sometimes occurs (FIGURE 4)



FIGURE 8 Photomicrograph of paraffin section of comparable portion of hair follicles as in FIGURE 7 showing hyperkeratosis and the formation of intrafollicular microabscesses

However, the presence of the virus usually appears to elicit only cellular hyperplasia and cells undergoing mitosis, despite the presence of cytoplasmic distemper inclusions, can be found (FIGURE 5). This increased activity leads to the piling up of cells in the pulmonary alveoli with formation of giant cells. In the brain it produces hyperplasia of the ependymal cells and astrocytes sometimes with multinucleate forms and hyperplasia of blood vascular endothelium with apparently, the formation of new vessels (FIGURE 5). In the skin this stimulation to hyperplasia, marked hyperkeratosis and parakeratosis in the epithelium of the skin and adnexal glands is a characteristic picture of canine distemper (FIGURES 7 and 8).

Summary

Canine distemper affects Canidae and certain other Carnivora. The virus is transmissible to chicken embryos, and suckling mice and hamsters. There

is an immunological relationship to measles. The most distinctive feature of the cytopathogenicity are (1) presence of distinct inclusion bodies containing virus antigen in both the cytoplasm and the nucleus of affected cell and capability of the virus to invade most of the cell types of the body.

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PROPAGATION CYTOPATHOGENICITY, AND HEMAGGLUTINATION HEMADSORPTION OF SOME ARTHROPOD BORNE VIRUSES IN TISSUE CULTURE

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Tissue culture methods have been employed for the cultivation of arthropod borne viruses for many years. One of the classic examples is the propagation of yellow fever virus by Theiler and his associates^{1, 2} in chick embryo tissue culture. At the present time more than seventy arthropod borne viruses have been isolated. Certain of these agents are antigenically related and have been placed by Casals and Brown in distinct serologic groups A, B, and C^{3, 4} by means of hemagglutination inhibition, complement fixation, and neutralization tests. In addition while there is already indication of other groups a large number of viruses still remain ungrouped.

Recent tissue culture investigations have been concerned chiefly with the study of some of these agents belonging to groups A and B.^{5, 6} During the past two years I have attempted the propagation of about 20 arthropod borne viruses some of which belong to groups A, B, and C and a few of which are ungrouped. The aim has been to determine conditions under which yield of virus is high and the presence of virus easily and regularly detectable.

METHODS OF STUDY

Viruses

TABLE 1 lists the viruses used and the tissues that were employed for their growth. The following viruses were successfully propagated: Group A Chikungunya,⁷ Eastern equine encephalomyelitis (EEE),⁸ Mayaro,⁹ Semliki Forest, Sindbis,⁸ Western equine encephalomyelitis (WEE). Group B Dengue Type 2, Japanese B encephalitis (JBE), Kyasanur Forest disease (KFD),¹⁰ West Nile (WN). Group C^{4, 5} Murutuba (Be An 15), Oniboca (Be An 17), Apeu (Be An 848), Murutucu (Be An 974), Caraparu (Be An 3394). Other viruses: Bwamba,² Be H 5056, Be H 151, Be Ar 671, and Be Ar 278.

Many of these agents are well known. I shall therefore briefly describe only the unfamiliar viruses. Of Group A Chikungunya virus isolated from arthropods and man in East and South Africa causes a denguelike disease. Mayaro virus isolated in Bolivia, Brazil, and Trinidad produces a systemic disease in man. Of

India is a virus. In

disease. Group C is composed of six viruses that have been isolated in Belém area of the Amazon region. Of the other viruses Bwamba produces a systemic disease in man.

were isolated in Belém by O. R. Causey. As yet they are not described.

Cell Cultures

As growth of virus may be dependent on the type of cell used, chick embryo cells as well as several different human cell lines were chosen. Cultures of whole, 9 day-old chick embryos were prepared by the method described by Dulbecco²². The mammalian cells were maintained as described in TABLE 2. In the course of preliminary experiments, it was observed that the addition of tryptose phosphate broth (TPB)²³ to the growth medium apparently enhanced

TABLE 1
VIRUSES CELL SYSTEMS

Group	Virus	Chick embryo		HeLa TPB		S. HeLa		Detroit-6		Human embryonic intestine	
		I*	CPE†	I	CPE	I	CPE	I	CPE	I	CPE
A	Chikungunya	0	0	+	+	-	-	-	-	-	-
A	EEE	+	+	-	-	-	-	-	-	-	-
A	Mayaro, Tr 4675	0	0	+	+	+	+	-	-	-	-
A	Mayaro, Tr 15337	+	0	+	+	+	+	-	-	-	-
A	Semliki Forest	+	+	-	-	-	-	-	-	-	-
A	Sindbis	+	+	-	-	-	-	-	-	-	-
A	WEE	+	+	-	-	-	-	-	-	-	-
B	Dengue Type II	0	0	+	0	+	+	-	-	-	-
B	JBE	+	0	+	+	-	-	-	-	-	-
B	KFD	+	0	+	+	+	+	+	+	+	+
B	WN	+	+	-	-	-	-	+	+	-	-
C	Marituba (Be An 15)	0	0	+	+	-	-	-	-	-	-
C	Oriboca (Be An 37)	+	0	+	+	-	-	-	-	-	-
C	Apeu (Be An 848)	0	0	+	+	+	0	-	-	-	-
C	Murutucu (Be An 974)	0	0	+	+	-	-	-	-	-	-
C	Carsparu (Be An 3394)	0	0	+	+	+	0	-	-	-	-
Other	Bwamba	+	+	-	-	-	-	-	-	-	-
Other	Be H 5056	0	0	+	0	0	0	-	-	-	-
Other	Be If 151	0	0	+	0	+	0	-	-	-	-
Other	Be Ar 071	+	0	+	0	0	?	-	-	-	-
Other	Be Ar 278	+	0	0	0	0	6	-	-	-	-

* Infectivity

† Cytopathogenic effect

Key + test for infectivity positive or cytopathogenic effect present 0, no viral multiplication or no cytopathogenic effect -, not tested

the rate of cellular multiplication of HeLa cells. Our HeLa TPB cell line has undergone more than 180 transfers and has proved especially useful for the growth of some of the viruses. For all studies employing mammalian cells, a maintenance medium consisting of 97 per cent of Eagle's medium²⁴ and 3 per cent of calf serum supplemented with antibiotics (100 units of penicillin, 100 µg of streptomycin, and 25 µg of mycostatin per 1 ml) was introduced before viral inoculation.

Propagation and Cytopathogenicity

As can be seen from TABLE 1, every virus tested multiplied in at least one of the cell systems and often in more than one. Presence of virus was detected by intracerebral inoculation of mice with the fluid phase of infected tissue cultures

TABLE 2
TISSUE CULTURE SYSTEMS

Cell culture	Source	H ^a	CFE ^a	Growth fluid				Transfer intervals (days)	No. of cells per tube
				HOS ^c	E ^c	HAS ^c	TPB ^c		
Chick embryo	Div. of Laboratories and Research Albany N.Y.	90	5	5		20	15	7	5×10^6 2×10^6
HeLa TPB		65							
S ₁ HeLa	T. T. Puck via A. Moore Sloan Kettering Institute New York N.Y.				80	20		7	2×10^6
Detroit 6	A. Moore Sloan Kettering Institute New York N.Y.				80	20		7	2×10^6
Human embryonic intestine	G. Henle via Div. of Laboratories and Research Albany N.Y.				80	20		7	2×10^6

^a Containing 100 units of penicillin 100 µg of streptomycin and 25 µg of mycostatin per ml.

Key: H Hanks balanced salt solution adjusted to pH 7.4 to 7.6 CEF chick embryo extract HOS horse serum F Eagle's medium HAS human adult serum TPB tryptose phosphate broth

TABLE 3
CYTOPATHOGENIC EFFECT IN CHICK EMBRIO TISSUE CULTURE

Group	Virus	Titer* inoculum mouse brain (10 ⁶ c)		Number of passages	CPE†	Titer* current passage (fluid phase)	
		LD ₅₀ (1 ml)	CPD ₅₀ (1 ml)			LD ₅₀ (1 ml)	CPD ₅₀ (1 ml)
A	EEE	10.3	9.7	25	3+	8.0	8.0
	Semliki Forest	9.0	8.5	6	3+	7.8	8.7
	Sindbis†	7.2	7.0	29	4+	7.4	8.0
	WFR	9.2	9.3	5	3+	8.8	8.5
B	West Nile†	9.1	5.5	31	3+	7.8	8.3
	Other virus	7.3	2.5	25	3+	5.2	5.5

* TABLE 3 and TABLES 4 to 9
and granulation of scattered cells
† cells 4+, as above but most

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WN and Bwamba viruses became well established only after several transfers. Onset of cellular injury was rapid for group A and usually well established by the second day. In group B, changes were first noticeable between the second and third days postinoculation, while Bwamba virus was even slower in that well-defined focal necrotic areas of cells (microplaques) preceded diffuse involvement of the monolayer. All agents discussed in TABLE 3 were easily propagated when inoculated into freshly seeded tubes containing a massive cell inoculum of 5×10^6 cells/ml.

With the viruses listed in TABLE 3, CPE in chick embryo tissue cultures was distinct and represented a reliable criterion for viral multiplication. The specificity of the CPE was ascertained by neutralization tests using immune monkey sera. The last two columns of TABLE 3 show that CPE was just as reliable as the production of deaths in mice for indicating the presence of virus. Titer values in this and subsequent tables have been converted to values corresponding to 1 ml of test material.

TABLE 4
PLAQUES CHICK EMBRYO TISSUE CULTURE

Group	Virus	Inoculum	Titer			Days	Boundary of plaque	Diameter (mm)
			LD ₅₀ (ml)	CFD ₅₀ (ml)	PFU (ml)			
A	WFF	Mouse brain	10.1	9.5	4×10^6	3	Sharp	6
	WEI	Fluid phase 6th tissue culture passage		7.0	4×10^6	2	Sharp	6
A	Semliki Forest	Fluid phase 2nd tissue culture passage	8.1		1×10^6	3	Sharp	6
B Other virus	West Nile	Mouse brain	9.0	6.6	8×10^6	5	Sharp	3.4
	Bwamba	Fluid phase 12th tissue culture passage	5.8	4.5	2×10^6	8	Sharp	3

Assuming that the ability of a virus to produce cellular necrosis or injury is a prerequisite for the formation of plaques, WFF, Semliki Forest, WN, and Bwamba viruses were studied in chick embryo cells by Dulbecco's plaque technique.¹¹ The results are shown in TABLE 4. Plaques with sharp boundaries were obtained either with infected mouse brain suspensions or with the infectious fluid phase of tissue cultures (1 ml of virus dilution per 10 ml bottle one hour adsorption at 37° C.).

10^6 infectious mouse brain doses) were employed. CPE occurred in all instances as of the very first passage and was constant thereafter. With the exceptions of JBF virus and KID virus, complete destruction of monolayers was observed. Successive passages were carried out as soon as slight cellular

changes became visible, at short intervals of 3 to 4 days. Microscopically distinct areas of focal cellular necrosis (microplaques) progressing to complete involvement of the monolayer were characteristic for Chikungunya, Mayaro, and viruses of group C. This indicates that Dulbecco's plaque technique in all probability, could be used successfully with these agents. FIGURES 1 to 4 show Giemsa stained²⁵ clonal S₂ HeLa cells as well as HeLa TPB cells inoculated with Mayaro virus, strain Tr 15537, and Oriboca virus, respectively. The focal nature of the initial lesion is evident especially from FIGURE 3. In cultures infected with the newly discovered KFD virus and with JBE virus, injury was more diffuse. Early lesions consisted of rounded and granulated cells scattered throughout the monolayer. Plaques with poorly defined edges

TABLE 5
CYTOPATHOGENIC EFFECT IN MAMMALIAN CELLS

Human cell line	Group	Virus	Log of infectious mouse brain doses inoculated at start of experiment	Number of passages	CPE*	Titer of flu phase of infected tissue cultures	
						LD ₅₀ (1 ml)	CPD ₅₀ (1 ml)
S ₂ -HeLa	A	Mayaro	5.1	16	4+	6.3	7.2
S ₂ -HeLa	A	May Tr 15537	6.2	16	4+	6.8	7.4
S ₂ HeLa	B	Kyasanur Forest Disease	6.7	25	3+	8.0	7.8
HeLa TPB	A	Chikungunya	4.6	10	4+	6.0	7.1
HeLa TPB	B	Kyasanur Forest Disease	6.7	50	3+	8.0	8.5
HeLa TPB	B	Japanese B encephalitis	4.7	6	2+	5.8	6.0
HeLa TPB	C	Marituba (Be An 15)	5.0	10	3+	5.9	6.3
HeLa TPB	C	Oriboca (Be An 17)	6.5	10	4+	6.5	7.0
HeLa TPB	C	Murutucu (Be An 974)	6.5	10	4+	5.9	7.0

* CPE see footnote, TABLE 3

have been described by Bhatt and Work¹⁴ in monkey kidney cultures inoculated with JBE virus.

Hemagglutination Hemadsorption

During experiments designed to propagate KFD virus in chick embryo tissue cultures it was found that, although a large amount of virus was produced as

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as a means of virus detection in tissue cultures inoculated with

mumps and croup associated (CA) viruses. Their findings encouraged us to search for a similar phenomenon in cultures inoculated with KFD virus. We were successful only when pH conditions similar to those described for the conventional hemagglutination (HA) and hemagglutination inhibition (HI) test by Clarke and Casals²¹ were maintained. To achieve this we used the same chemical reagents and insofar as possible, the same techniques that they recommend²¹. At the present time a positive hemagglutination hemadsorp-

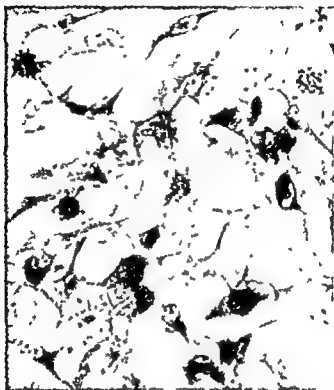


FIGURE 1. Clonal 50 Hela cells inoculated with Mayaro virus strain Tr 15537 harvested 24 hours after inoculation and stained by Giemsa's method. Note contraction, rounding and granulation of cells. Increase in cytoplasm basophilic (dark blue or granulate). $\times 500$.

tion test in tissue culture (THA) has been obtained for the following viruses: WFE, Sindbis, KFD and WN.

The THA test for KFD virus is routinely performed as follows. The fluid phase of infected and uninfected cultures is removed. The cellular monolayer is washed once with 1 ml. of Hanks balanced salt solution adjusted to pH 7.4 to 7.6. The washing fluid is discarded and borate saline (pH 9.0) is added (0.2 ml. per culture). After incubation at 37°C. for 30 min. goose erythrocytes suspended in virus adjusting fluid to give a final pH of 6.4 (considered opti-

mal for KFD virus) are introduced 0.2 ml per culture and mixed well. For optimal results the optical density of the red cell suspension is higher than the one used in the HA or HI test and corresponds roughly to a dilution 1:11 of the standardized cell stock²¹ or approximately is equal to a 1 per cent suspension of packed cells and has an optical density of 1.8. Following a second incubation of 1 hour at 4°C, tubes are drained for 5 min. and the test is read immediately. FIGURES 5 to 7 representing unstained clonal S₂ HeLa cells infected with the

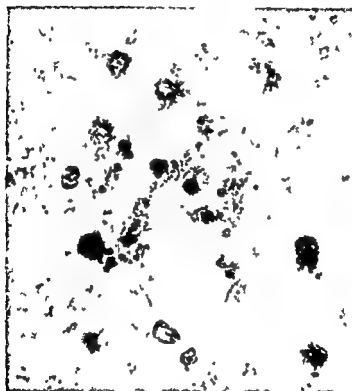


FIGURE 2. Clonal S₂ HeLa cells un inoculated stained by Gemsa's method. Monolayer intact. X570.

forty-ninth passage of KFD virus show, in addition to CPE, the characteristic findings of the THA test. The specific sign for presence of viral hemagglutinin is an end to end agglutination of the nucleated goose erythrocytes. From 3 to 12 red cells are linked together longitudinally. Such a unique arrangement of red cells has been observed only in infected cultures and is not seen in uninfected preparations. This chain formation can be inhibited specifically by

In a positive THA test the
no other chains appearing then
some attached around isolated

cells or groups of cells horseshoe formation or rosette formation occurs At



cells or to tube wall. The length of the red cell chains depends on the optical density of the red cell suspension and on the amount of viral haemagglutinin. If the optical density of the added red cell suspension is reduced to 0.75, a value used in the plate HA and HI test, chains are shorter, double and triple chains are rare and rosette formation is usually lacking. Readings of THA tests have been based exclusively on chain formation. In scoring the results the following system was used: + chain formation (3 or more erythrocytes), ± occasional chains (2 to 3 erythrocytes) and 0 no chain formation. 10

tests it must be shown that uninoculated controls are completely free of chain formation

The quantitative aspects of the THA test as a means of detecting virus multiplication were then tested as summarized in TABLE 6 and in FIGURE 8. KFD virus was propagated for many passages in chick embryo tissue cultures. HeLa TPB cells, clonal S₂-HeLa cells, Detroit 6 cells, and human embryonic intestinal cells. Viral hemagglutinin was produced in all systems. Fluid phases of in

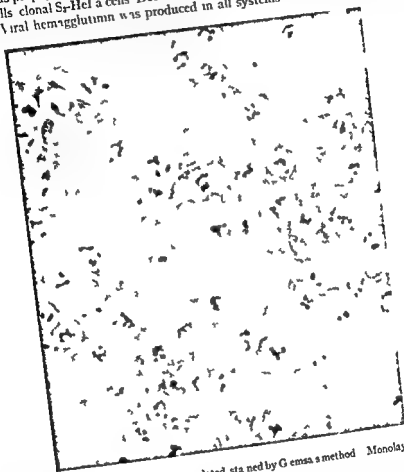


FIGURE 4. HeLa TPB cells uninoculated stained by Giesma's method. Monolayer intact. X570

fectured cultures were titrated in mouse and tissue cultures and LD₅₀ titers were then compared with CPD₅₀ (cytopathogenic effect doses₅₀), THD₅₀ (tissue culture hemagglutination hemadsorption doses₅₀) and TID₅₀ (tissue culture infectious doses₅₀). The latter figure was obtained by mouse infectivity tests with the undiluted fluid of cultures inoculated with different virus dilutions. The values listed in the two last columns of TABLE 6 indicate a good correlation between THD₅₀ and TID₅₀. This fact is of great importance for the cultivation of KFD virus in chick embryo tissue cultures where CPE is absent in

spite of a very high TID₅₀. Whenever viral hemagglutinin was present, infectious virus was present, too. As another approach to correlating THA and infectivity, a very small amount of KFD virus about 100 THD₅₀ (forty-fourth passage of KFD virus in chick embryo suspension culture), was inoculated into freshly seeded tubes containing 5×10^6 chick embryo cells/ml, thus ensuring the greatest opportunity for contact between the cell population and the small virus inoculum. Appearance of infectious virus in the fluid phase was studied at different times by mouse infectivity tests and THA tests in

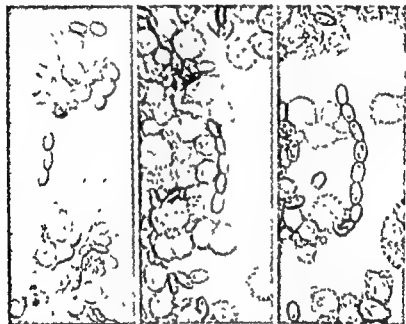


FIGURE 5. Chick embryo cells inoculated with KFD virus harvested 48 hours after inoculation, unstained. Sections of 3.5 and 7 hours longitudinally agglutinated nucleated erythrocytes. Considerable pathogenic effect. $\times 355$.

chick embryo tissue cultures (FEBRIF 8). The slopes of the curves are very similar indicating that the THA test is as valuable a means for the detection of virus as the mouse inoculation technique.

1.2.1. Antibodies in Tissue Culture

Neutralization test. Cell: Murutucu virus Be In 074 (Group C). The ability of Murutucu virus to lyse HeLa TPB cells was utilized in the assay of antibodies in a hyperimmune mouse serum. The results of the experiment are shown in TABLE 7. Serially diluted sera of un inoculated and of vaccinated mice were tested at the same time. Equal amounts of undiluted serum mixed with varied concentrations of virus suspensions (tenth passage of Murutucu



FIGURE 6 Clonal S₂-HeLa cells inoculated with KFD virus harvested 48 hours after inoculation unstained. Note chains of red cells attached to single infected cells. Horseshoe format on. X355.

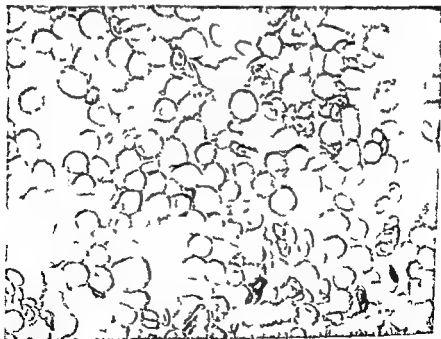


FIGURE 7 Clonal S₂-HeLa cells un inoculated control preparation unstained. THA test negative. A few red cells trapped mechanically. X355.

virus, Be An 974 in HeLa TPB cells fluid phase) were incubated at 37° C for 1 hour, then inoculated into mice intracerebrally in 0.03 ml amounts and into tissue cultures in 0.1 ml amounts. Results were clear cut. In both cases complete neutralization was obtained with all dilutions tested.

TABLE 6
KAYANUR FOREST DISPERSE COMPARISON OF ID₅₀ IN MICE
TCPD₅₀, THD₅₀* AND TID₅₀†

Culture	Passage	LD ₅₀ (1 ml)	TCPD ₅₀ (1 ml)	THD ₅₀ (1 ml)	TID ₅₀ (1 ml)
Chick embryo	5	>70	15	7.5	7.5
Chick embryo	15	7.3	<1	8.5	8.4
Chick embryo	20	7.3	<1	8.3	8.3
Chick embryo	37	7.3	<1	8.1	8.1
HeLa TPB	5	7.5	5.8	6.5	6.5
HeLa TPB	10	7.8	7.2	6.5	6.5
HeLa	5	8.3	7.2	6.5	6.5
HeLa	10	7.8	6.5	7.3	7.3
HeLa	15	8.0	7.2	7.8	7.8
HeLa	25	8.7	8.0	7.2	7.2
HeLa	20	8.0	7.8	7.5	7.5
HeLa	10	8.8	Incomplete	7.2	7.2
Detroit 6		7.2	7.5	7.5	7.5
Human embryo					
intestine					

* THD₅₀ tissue culture hemagglutination hemadsorption D₅₀
† TID₅₀ tissue culture infectious D₅₀

KFD virus - chick embryo suspension culture

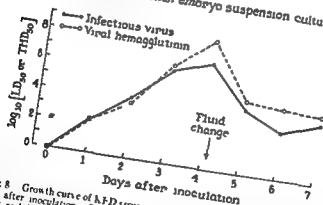


FIGURE 8. Growth curve of KFD virus in chick embryo tissue culture. Medium changed four days after inoculation. In this figure titers are expressed as log₁₀ LD₅₀ per 1 ml (●) and log₁₀ THD₅₀ per 1 ml (○) of pooled fluids of three tubes. The asterisk indicates the titer of the original inoculum. Pooled fluids were titrated in a bulk in chick embryo tissue cultures. LD₅₀ 10⁶ or THD₅₀ 10⁶ and rates no detectable virus.

Neutralization test (THI) KFD virus (Group B). Sera of young adult hamsters inoculated intraperitoneally with about 10⁶ infectious doses of KFD virus were collected 1 month postinoculation and tested for neutralizing antibodies. Sera of uninoculated hamsters were collected and tested at the same time. The test was carried out as follows: unaltered sera were mixed with

100 LD₅₀ of KFD virus (10 per cent infused mouse brain diluted 10⁻⁵) and incubated for 1 hour at 37° C. The mixtures were then inoculated into chick embryo tissue cultures (0.1 ml), as well as intracerebrally into adult mice (0.03 ml). Six tubes or six mice, respectively, were used for each mixture. Due to occurrence of nonspecific deaths, the number of mice was less than 6 in some instances. Parallel titrations were performed in both tissue cultures and mice at the same time. Mice were checked for the occurrence of deaths every day for 15 days and the average survival time (AST) calculated. Chick embryo tissue cultures were tested for the presence of viral hemagglutinins by the THA test on the fifth day after inoculation. Results of this experiment are shown in TABLE 8. Viral hemagglutinin was detected in all tubes inoculated with mixtures of virus and sera devoid of antibodies. In tubes inoculated with mixtures of virus and sera from immune animals, the virus was neutralized and THA tests were negative. There were no discrepancies between the tissue culture and mouse neutralization tests, the former evaluated on the fifth day as compared to the latter, which was terminated 15 days postinoculation.

TABLE 7
GROUP C NEUTRALIZATION OF MURUTUCU VIRUS

Virus* dilution	Normal mouse serum		Hyperimmune mouse serum	
	Infant mouse (intracerebrally)	HeLa TPB cells	Infant mouse (intracerebrally)	HeLa TPB cells
10 ⁻¹	7/7	3/3	0/8	0/3
10 ⁻²	7/7	3/3	0/8	0/3
10 ⁻³	7/7	3/3	0/8	0/3
10 ⁻⁴	1/7	2/3	0/8	0/3
10 ⁻⁵	0/7	0/3	0/8	0/3
Titer†	6.1	6.3	<2	<2

* Source of virus: tenth passage of Murutucu virus in HeLa TPB cells fluid phase

† Expressed as log₁₀ 1/ml of test material

Hemagglutination hemadsorption inhibition test (THI) KFD virus (Group B)
In these experiments sera of 6 laboratory workers, of 2 rabbits immunized against KFD virus, as well as of 1 normal rabbit were tested by conventional HI test as well as by the THI test. All sera were treated with kaolin and adsorbed with goose erythrocytes²¹. The same conditions were necessary for success in both methods. Five days postinoculation of 100 THD₅₀ into chick embryo tissue cultures, a THI test was performed as follows: Treated sera, diluted 1:10 in borate saline (pH 9.0) were added to washed cultures, 0.2 ml diluted 1:10 in borate saline (pH 9.0) were added to washed cultures, 0.2 ml

C, tubes

TABLE

9 shows a comparison of results obtained by the HI test with those from the

TABLE 8
GROUP B NEUTRALIZATION OF KFD VIRUS IN CHICK EMBRYO TISSUE
CULTURE (THA) AND BY INTRACEREBRAL MOUSE TEST

Sera	Virus dilution (10% mouse brain)	THA test*						Intracerebral mouse test			
		No of tubes					Result	Died	Survived	AST (days)	Result
		1	2	3	4	5					
Hamster 1 N	10 ⁻⁵	+	+	+	+	+	6/6	6	0	6.5	Neg
2 N	10 ⁻⁵	+	+	+	+	+	6/6	6	0	6.3	Neg
3 N	10 ⁻⁵	+	+	+	+	+	6/6	6	0	7.2	Neg
4 N	10 ⁻⁵	+	+	+	+	+	6/6	4	0	6.5	Neg
5 N	10 ⁻⁵	+	+	+	+	+	6/6	2	0	7.0	Neg
6 Imm	10 ⁻⁵	0	0	0	0	0	0/6	1	4	14.0	Pos
7 Imm	10 ⁻⁵	0	0	0	0	0	0/6	1	5	13.0	Pos
8 Imm	10 ⁻⁵	0	0	0	0	0	0/6	6	0	15.0	Pos
9 Imm	10 ⁻⁵	0	0	0	0	0	0/6	1	4	13.2	Pos
10 Imm	10 ⁻⁵	0	0	0	0	0	0/6	1	4	13.6	Pos
Control		0	0	0	0	0		ND	ND		
Virus titration	10 ⁻⁵	+	+	+	+	+	6/6	5	0	6.6	
	10 ⁻⁶	+	+	+	+	+	6/6	4	0	7.7	
	10 ⁻⁷	+	+	+	+	0	4/6	4	1	10.0	
	10 ⁻⁸	0	0	+	0	0	1/6	0	5	15.0	

Titer † THD₅₀ = 84LD₅₀ = 89

* Scoring explained in text.

† Expressed as log₁₀/1 ml. of test material

TABLE 9
GROUP B THA TEST WITH KFD VIRUS IN CHICK EMBRYO TISSUE CULTURE

Sera	History	Tissue culture* THA test	Plaque titers
Human, W R	No vaccine	0	0
Human, C M	RSSE vaccine	4	3
Human, N D	RSSE vaccine	0	0
Human, S W B	RSSE vaccine	3	0
Human, D N D	Laboratory infection with KFD virus	3+†	4
Human, R C	Laboratory infection with KFD virus	3+	7
Rabbit, No 1	KFD—mouse brain susp	8	7
Rabbit, No 2	KFD—mouse brain susp	6	8
Rabbit, No 3	Uninoculated	0	0

* THD₅₀, 10⁻⁴ 1/1 ml on day of test

† Plate test performed with 8 units of hemagglutinin

‡ End point not reached.

the immunizing agent. The only discrepancy occurred when sera of laboratory workers vaccinated with formalized Russian Spring Summer encephalitis (RSSE) virus were tested for HI antibodies against KFD. As has been pointed out, KFD virus is serologically closely related to the RSM virus. Also it must be remembered that in the HI test 8 units of hemagglutinin were used as contrasted with 1.5×10^6 THD₅₀/ml viral hemagglutinin doses determined

on the day on which the THI test was carried out. Since there is no reason to assume that the amount of antigen is the same in such different tests as the HI and THI, it is perhaps, remarkable that the degree of correlation was so close

SUMMARY

Arthropod borne viruses are easily propagated in either chick embryo tissue cultures or mammalian cell lines

Viral infectivity can be measured by direct observation of cellular alteration (CPE) or by production of viral hemagglutinin as measured in the tissue culture hemagglutination hemadsorption (THA) test

Assays of antibodies either by neutralization tests or by tissue culture hemagglutination hemadsorption inhibition (THI) tests appear to correlate reasonably well with results obtained in mouse protection tests or HI tests

Tissue culture appears to be an effective device for the cultivation and assay of a large group of arthropod borne viruses and the detection of their antibodies

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BIOCHEMICAL STUDIES OF VIRUS INFECTED CELLS*

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The orientation imposed by the subject of this monograph leads us to emphasize those aspects of the problem which are of greatest importance to the understanding of the virus infection process.

the ensuing chain of secondary reactions and the translation of molecular alterations to macrochanges together, comprise the scope of our interest. Other papers in this monograph express pre-eminently how of any viral infection can be in general terms the over all pattern as it appears in the light of present obser-

the chemistry of the virus has waited upon developments in tissue culture that allow first of all the important separation of the primary cytopathology from the inflammatory and proliferative effects of a secondary nature. An equally important result of the tissue culture technique is the ability to observe changes most easily by comparison of infected and normal cultures where, because of the completeness of infection one is really comparing normal and infected cells. Only preliminary studies of the rate and nature of the initiation of infection allow one to proceed with assurance in this regard.

A variety of techniques is available to limit the infection in a particular culture to a single sequence. This is an essential for the correlation of viral synthesis with the observed cellular changes and for the determination of the natural order of these processes. If classic methods of biochemistry are to be employed the use of mass cultures becomes mandatory and of greater value where infection is initiated synchronously in all cells since this provides for interpretation at the cellular level. The few results already available emphasize the value of complete time studies during the infectious cycle if we are to have confidence that important observations are not overlooked.

Thus with present techniques it is possible to direct our observations to very uniform multicellular cultures in which nearly every cell is undergoing a single cycle of infection in an almost synchronous manner. However what is desired are cultures in which all cells during their growth cycle are performing physiological and biochemical activities synchronously.

Also prerequisite to a determination of effects whose cause is viral is a clear notion of the properties of the normal cell these properties must be definable and measurable.

Inductive or stimulatory effects are best considered in resting cell systems.

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wherein activities are only potentials and in which the environment is competent for the realization of the potentials. Success in provoking a particular activity by virus or any other instrument establishes the competency of environment for this activity, failure only leaves the question unanswered.

Inhibitory or blocking effects of virus, in contrast, are best studied with cells in a dynamic state where the activities in question are proceeding. Properties of cells manifest only in the dynamic state are elegantly followed in synchronized cultures or in single cells.

Observations that fall in the first category of stimulatory or inductive effects will be considered first. Many of the findings presented elsewhere in monograph are appropriate here and could serve as examples. However, because I have greater familiarity with the details of the studies reported by H. F. Maassab, I shall emphasize these preferentially.

Maassab reported that the composition of his control cultures during the experimental period remained constant when considered on a cellular basis. Constant composition would result if a static situation existed in which no net synthesis of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) was occurring or equally as well if synthesis was proceeding rapidly in cells also undergoing completely asynchronous division.

Careful study of cellular division in cultures of ordinary HeLa cells sustained in 90 per cent maintenance solution (Scherer, 1953) and 10 per cent equine serum indicates that the former situation prevails. In the maintenance medium cellular increase does not occur, net synthesis of cytoplasmic RNA, nuclear RNA, and DNA does not proceed at detectable levels although these components may be metabolically active as indicated by the considerable radioactive phosphate that they incorporate. The cells, of course, are utilizing glucose, consuming oxygen, and producing acid. Upon replacement of the maintenance medium with 90 per cent Eagle's medium (Fagü, 1955) and 10 per cent equine serum, cellular division resumes, the cells grow and increase in number. At present it has not been determined whether the maintenance medium either is simply deficient or is inhibitory; however, in either case, the effects are multiple, although possibly secondary to a primary or singular defect.

When such cultures of resting cells, sustained in maintenance medium, are infected with poliovirus, as Maassab has described, copious quantities of RNA and protein accumulate in the cytoplasm. It follows therefore, that in the system precursors of RNA and protein and an appropriate source of energy are available. In the resting cell these syntheses are potential, the environment is

phase does not occur
esis of DNA or nuclear
ce medium is compati-
syntheses or ex

RNA, it is not possible to decide whether it is compatible with these functions or not. Conclusions cannot be drawn as to whether the virus, on infecting the cell, is able to sti-

can inhibit them. The competence of the environment for DNA synthesis might be established by infecting the cell with some other virus (Newton and Stoker, 1958) or the question might be examined experimentally, with cells in a growth medium. As yet, these experiments have not been completed. It should be emphasized, however, that there are distinct early metabolic, as well as morphologic, effects demonstrable in the nucleus of infected cells.

So much for the basic description of the normal resting cell and its potential activities. The next consideration is one of the viral effect that has been described: the hyperdevelopment of cytoplasmic elements (Maassab *et al.*, 1957, Loh *et al.*, 1958).

The synthetic activity induced in the resting cell by the infection may not exceed that of the ordinary cell during the growth phase, particularly if we consider that the RNA and DNA of the daughter cell probably are formed only during a portion of the interphase and that the total generation time is only eighteen hours. One need not assume that the virus increases the basic potential of the cell.

The effect here of virus is conceived most simply (and this is speculation) as an uncoupling or disengaging action that releases the cytoplasmic reactions from obligate cross linkages with other major synthetic activities with which they are normally coordinated.

The constant quality of "..."

operation during cellular

synthesis of various species of RNA could not proceed independently of others. When conditions in a particular locus of the cell are suitable for RNA synthesis, all templates in the area function and, possibly by simple competition, duplicate in fixed proportions.

Again, it is economical to propose that this normal coordinating principle operates after infection and that replication of the viral specific species of RNA triggers the development of other templates of RNA already present in the host cell.

It is obvious that we do not understand the principle underlying the coordinated synthesis of the various RNA molecules in the ordinary cell and much less how the virus may participate under such a principle. One clue may be the fact that the infectious cycle can be initiated by the isolated RNA of the poliovirus. If we restrict our thinking to the nucleic acid, it is tempting to propose that in the ordinary cell nucleic acid synthesis occurs only at those times when the nucleic acid is freed from the nucleoprotein. Perhaps the simple presence of an active or free nucleic acid molecule in the metabolic milieu is sufficient to catalyze the dissociation of the resident nucleoproteins. In one sense the action of the virus would be to condition certain areas of the cell to

that was not virus and not destined to be virus but rather was a mere *prophage* (the quantities of materials

It is quite clear from experimental evidence (the quantities of materials involved, their distribution among the subcellular components, and the base

with adenovirus (Boyer *et al*, 1957). The second point, regarding the cellular

plasm. This corresponds to fraction III, which we have found to increase in total protein content (Loh *et al*, 1958).

At this point we should not fail to recall that well authenticated examples can be cited also in which there is the induction of synthesis of materials characteristic of infection, but of neither viral nor cellular nature. I refer to the proteins comprising the crystalline matrix found in cells of insects with polyhedral diseases (Bergold, 1953), the hemagglutinating protein of vaccinia infection (Chu, 1948), and the nonviral antigens found after phage infection of *Escherichia coli* (Watanabe, 1957). For these one would like to reserve some special functions in the viral developmental process. These may be of structural or, as with the T₂ bacteriophages, of metabolic significance (Ellis and Cohen, 1957).

A third class of materials, not to be ignored in preparing a balance sheet for infected cells, is that comprised by products resembling virus but lacking full biological activity. This includes all the well established soluble antigens and so-called incomplete virus forms. These are of such extensive and diverse natures that it would not serve our purpose to review them here.

It suffices to say that with poliovirus infection and, possibly with the others cited, the first class of newly formed materials is quantitatively of the greatest significance and accounts for much of the histochemical visualization.

The central problem is: What is the biochemical basis of the cytopathogenic effect, how can any of the considerations just outlined be related to the central problem?

From a teleological viewpoint, there would seem to be value in the suppression of limited phases of cellular development under conditions restricting the full development of the cell.

With the HeLa cell two examples may be cited: the action of X irradiation in the presence of thymine, and the action of thymine in the presence of X irradiation.

ever, once unbalanced development has occurred in the presence of inhibitor, viability cannot be restored upon the addition of thymine. The change is irreversible.

RELATIONSHIP OF VIRAL ANTIGENS TO INCLUSION BODIES

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The technique of labeling antibodies with fluorescein, as developed by Coons *et al*,^{1, 2} has become a very useful tool for immunohistochemical studies. It has a particular advantage in the study of viral infections because the antigens may be localized precisely in an infected cell by this relatively simple procedure.

The antibody molecules in a specific serum can be labeled with fluorescein and used as a direct histochemical stain for antigens. Many viruses such as influenza, mumps, vaccinia, fowl plague, Egypt infectious canine hepatitis and canine distemper have been detected by this direct staining method in tissue sections, as well as in infected cells from tissue cultures. On the other hand, unlabeled specific antiserum can be placed over tissue cells containing antigen. The antibody globulin molecules are deposited on the sites of antigen. After the excess of antibody has been washed away, the bound antibody molecules may be detected by fluorescein labeled specific antiglobulin

(4) the unlabeled serum can be diluted for antibody titrations. Measles, varicella, primary atypical pneumonia, herpes simplex, poliomyelitis, psittacosis and others have been successfully studied in this way.

It is obvious that fluorescent antibody staining will help to elucidate the question of whether inclusion bodies represent aggregates of viral particles or are merely morphological residuum from viral infections. Several viruses that produce classic inclusion bodies have been investigated.

Infectious canine hepatitis is an acute disease of viral origin in dogs and is characterized by centrilobular necrosis of the liver. Histologically, large intranuclear eosinophilic inclusion bodies are seen in the hepatic parenchymal cells, as well as in the endothelial cells of smaller veins, venous sinuses, and liver sinusoids. The typical Type A inclusion bodies had been noted by Cowdry and Scott³ in 1930 in two experimentally infected dogs. However, the disease was first described under the present name by Rubarth⁴ in 1917. Coffin *et al*,⁵ using the direct method of fluorescent staining demonstrated that the intranuclear inclusions in experimentally infected dogs contained a

hematoxylin and eosin. The nuclear membrane continued to contain viral antigen during the entire course of development of the inclusion bodies.

Canine distemper is a common infectious disease in dogs, foxes, and weasels.

The causative agent is a filtrable virus first discovered by Carré⁶ and later isolated by Laidlaw and Dunkin.⁷ Inclusion bodies in various body tissues of infected animals have been demonstrated frequently by staining with hematoxylin and eosin, by Seller's method,⁸ or by Shorr's method.⁹ When smears of urinary bladder epithelium from infected dogs were treated with specific fluorescein labeled anticanine distemper serum, numerous fluorescent masses were seen in the cytoplasm of the epithelial cells. After the smears were studied under the fluorescent microscope and photomicrographs were taken, the same slides were then stained by Seller's or Shorr's method. It was found that in

tory.¹¹ However, smears stained by Shorr's method (S-3 stain) showed interesting differences. The reddish inclusion bodies characteristic of distemper infection⁹ usually did not contain viral antigen. The fluorescent masses of viral antigens appeared faintly gray blue in the S-3 stains, and would often be passed unnoticed if they were not looked for specifically. This suggests a

ing on their stage of development.

The problem of whether the intranuclear eosinophilic Type A inclusions in herpes simplex infections contain virus particles has aroused the interest of many investigators. Cowdry¹² in 1930 found that the herpes inclusions did

clusions contain the nucleoprotein.

intranuclear inclusions do not possess such nucleoprotein. Gray and Scott,¹³ in their differential centrifugation experiments in herpes infected chick embryo liver, demonstrated that significant amounts of infectious virus could be recovered from isolated nuclear fractions of infected liver during the early stage of infection, while the nonnuclear fractions of virus increased as the infection progressed. Morgan *et al.*,¹⁴ by electromicroscopy, suggested that the initial site of herpes virus development is in the cell nucleus and subsequently is released into the cytoplasm, where it becomes mature. Using the fluorescent antibody and hematoxylin eosin staining methods, Lebrun¹⁵ studied the course of the development of herpes simplex inclusions in infected tissue culture of human epidermoid carcinoma cells. She observed that the detectable herpes antigen first appears as small fluorescent spherical spots in the nucleus, slowly increases in amount, and finally fills the whole nucleus. At this stage the clear antigenic material is basophilic and does not correspond to the Type A inclusions. Specific antigenic material gradually diffuses into the cytoplasm and increases in amount. At the same time the nucleolus decreases until it becomes very small or absent. Cells at this stage are devoid of typical Type A inclusions that contain little

From all the above data it is clear that, in the infected cells, herpes simplex virus multiplies in the nucleus, incorporates deoxyribose nucleoprotein¹⁴ assumes a central body 30 to 40 μ in diameter with a single membrane¹⁵ and is antigenic¹⁶. As the development progresses the virus is released from the nucleus to the cytoplasm and assumes a second outer membrane¹⁷ leaving the

bodies in brain smears or sections is the most helpful procedure beside actual isolation of the virus. Wolman and Behar¹⁸ in following the development of Negri bodies by the Feulgen reaction and by studying phosphatase and cholinesterase activities suggested that the inclusions represent aggregates of virus particles that multiply up to a certain limit and then diminish in number thereby resembling the events in morphogenesis of herpes inclusions. Goldwasser and Kissling¹⁹ have demonstrated that the Negri bodies in infected animal brains, as well as in salivary glands contain viral antigen. Although the studies were not carried out in staining with fluorescent antibody and other histological stains of identical cells the demonstration is sufficiently convincing because the fluorescent staining of the Negri bodies was inhibited by removal of the antibody with specific rabies virus. The authors suggested that this method may be useful in the diagnosis of Negri negative animals and in serologic assay of immunity in man or other animals undergoing rabies vaccination.

In summary the use of fluorescent antibody as an immunohistochemical stain has helped investigators to understand more about the problem of the relationship of viral antigens to inclusion bodies. In infectious canine hepatitis the intranuclear inclusions of this disease contain a high concentration of viral antigen that begins in the nuclear membrane and spreads to the interior of the nucleus. In canine distemper the inclusions may or may not contain viral antigen depending on the staining method used. In herpes simplex it is evident that viral antigen, presumably representing active virus is found in the inclusion bodies during the early stage of development. When the infection has run its course the virus is released from the intranuclear site of multiplication and leaves behind the eosinophilic inclusion as a cytological scar. More studies on the Negri bodies in rabies infection are needed. However it appears that they also contain viral antigen at some stages of their development.

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SOME PROBLEMS OF ELECTRON MICROSCOPY IN THE STUDY OF VIRUS-INFECTED CELLS*

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In the electron microscopy of virus infected cells, the problems are different from those found in the study of purified preparations of virus because the infectivity of particles visualized in cells cannot be tested directly by inoculation of animals or tissue cultures. In the absence of direct tests of infectivity, attempts at visualization of viral particles in cells have utilized comparisons of infected with uninfected cells. Under these conditions, however, it is possible that particles other than those of virus may appear as the result of viral action and may be mistaken for viral particles. For these reasons, we prefer to speak of viruslike particles, although it seems fairly sure in some instances that viral particles have been identified in cells. The effort to recognize viral particles in cells seems worth while, in spite of its difficulties and pitfalls, because of the insight that it may give into host parasite relationships. This paper is not intended as a review of the literature, but will point out problems that have arisen from our own work and exemplify some of the problems that must be dealt with in the electron microscopy of almost any kind of virus infected cells.

Our first electron microscopic observations were carried out with the lungs of mice infected with influenza virus, since earlier studies by light microscopy had shown numerous cytoplasmic inclusion bodies† in cells of the bronchial epithelium. The most pertinent facts from our early and recent studies by light microscopy may be summarized as follows. The inclusions were present after infection with several strains of Type A influenza virus of mice and with strains of virus that grow in the lung without producing pneumonic consolidation. Cytoplasmic inclusions were not present in the bronchial epithelium of uninfected mice infected with pneumonia virus of mice (PMV) or mice with pulmonary consolidation from Newcastle disease virus (NDV). Most of the inclusions were deeply basophilic but acidophilic inclusions were present also. Nuclei of the epithelial cells did not show stages of pyknosis, and specific silver stains for cilia indicated that ciliated cells were not destroyed by the infection. Observation of moving cilia in wet preparations confirmed the persistence of ciliated cells. Use of the method of fluorescence without exposure to antibody showed the inclusions showed autofluorescence without exposure to antibody (figure 1). Cells of uninfected bronchial epithelium did not phagocytize bacteria, and injection of bacteria into lungs infected with influenza virus did not show that the viral infection had. Additional evidence cells capable of phagocytosis.

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 † The Surgeon General, Department of the Army.

that the inclusions might consist of phagocytized nuclear debris was the finding that numerous inclusions developed during infection of mice that had received roentgen radiation of such a dose as to eliminate leukocytes from the viral lesion of the lung. Most of the inclusions gave a positive Feulgen reaction² (FIGURE 2), and the Feulgen reaction could be prevented by prior treatment³ of sections with desoxyribonuclease.*

Electron microscopy confirmed the presence of inclusion bodies in the cytoplasm and failed to show lesions of the nuclei. Sections of cells frequently permitted visualization of the nucleus with one or more inclusion bodies in the cytoplasm of the same cell. The inclusions contained particles that were estimated to be in the size range of influenza virus, and the suggestion was made



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Subsequent electron microscopy of cells infected with adenoviruses showed

form in size. The size of the particles in adenovirus infected cells was measured, and was only slightly less than that determined by other workers with other methods. In addition, the particles often occurred in crystal like arrangement and were shown to have internal structure resembling that of large viruses.⁶

This experience with viruslike particles in cells infected with adenoviruses

* Obtained from the Worthington Biochemical Co., Freehold, N. J.

led us to expect intracellular viruslike particles to be smoother, more discrete, and more uniform than the particles we had visualized in the cytoplasmic inclusions of epithelial cells infected with influenza virus. In addition, it was reported by Morgan *et al*⁶ that viruslike particles could be recognized on the cellular surfaces of chorioallantoic membranes of embryonated eggs infected with influenza virus. These workers also examined the lungs of mice infected

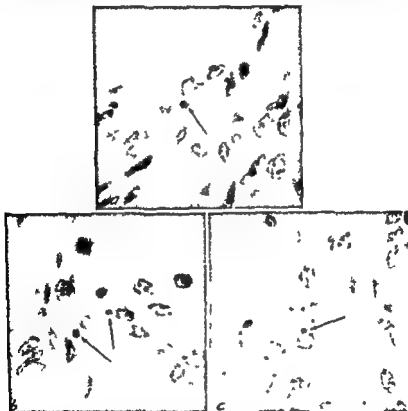


FIGURE 2. Bronchial epithelium of mice 40 hours after exposure to aerosol of the Weim strain of Type A influenza virus. Feulgen reaction without counterstain. Arrows indicate Feulgen-positive cytoplasmic inclusion bodies. $\times 9233$.

with influenza virus and thought that the cytoplasmic bodies were not composed of viral particles because the particles lacked uniformity and internal structure. They also found bodies with a similar appearance in chick embryo membranes infected with vaccinia (smallpox) and herpes viruses. A further reason for doubting that particles in the influenzal inclusions were particles of influenza virus arose from the findings of a number of workers concerning the nucleic acid present in purified preparations of influenza virus obtained from allanto-

fluid. Although virus from allantoic fluid may not necessarily have the same composition as that purified from mouse lung, it is noteworthy that highly purified preparations of influenza virus from allantoic fluid contain nucleic acid chiefly in the form of ribonucleic acid (RNA) and only trace amounts of deoxyribonucleic acid (DNA) or none at all^{7, 8}. Since the inclusions were primarily composed of particles giving a positive Feulgen reaction and since this reaction was prevented by prior treatment with desoxyribonuclease, it seemed probable that they contained large amounts of DNA and were unlikely to be viral particles. Our present tentative view, therefore, is that the cytoplasmic inclusion bodies induced by influenza virus in our experiments consist, to a large extent, of DNA accumulating in the cytoplasm as the result of viral infection.*

Estimation of the size of viruslike particles in infected HeLa cells had some value favoring their identification as adenoviruses, and we used polystyrene latex spheres as internal standards of measurement^{10, 11}. In this method the spheres of known size are sprayed on the surface of sections, and have the ad-

may be mentioned that fixation with osmium tetroxide causes some shrinkage of tissue and that sections through various levels of viruslike particles result in images that are usually smaller than the original particles because they do not go through the center. It is also known that latex spheres expand slightly from exposure to the beam of electrons¹².

The arrangement of particles in regular rows served as a distinguishing feature of the viruslike particles observed in the nuclei of HeLa cells infected with adenoviruses,¹ Morgan *et al*¹⁴ have shown that very large crystalline aggregates of viruslike particles occur. Such large crystals can be visualized by light microscopy¹⁵ and sections adjacent to those examined by electron microscopy have shown that the crystals give a positive Feulgen reaction¹¹. Since the viruslike particles are closely packed in the crystals, this evidence is an indication that adenovirus contains DNA. In this connection, it may be pointed out that nuclei of HeLa cells infected with Type 5 adenovirus contain particles that are clearly smaller than viruslike particles in uninfected cells, it would be

desirable to show that the particle itself had unequivocally distinguishing morphologic characteristics. The demonstration of internal structure in particles visualized in the nuclei of HeLa cells infected with adenoviruses has shown that they do have structure of considerable distinctiveness⁸ and the inner bodies surrounded by membranes resemble particles in cells infected with large viruses. Recently, we have sought to visualize the internal structure of adenoviruslike particles more clearly and have employed the method of Watson¹⁹.

* Sellers⁹ was unable to show greater than normal amounts of thymidylic acid in the lungs of mice infected with influenza virus.



FIGURE 3. Section of the nucleus of a HeLa cell 42 hours after infection with Type 3 reovirus. Electron micrograph shows internal structure of viruslike particles. The section was stained with uranyl acetate and covered with collodion. $\times 130,000$.

in which the sections are stained with uranyl acetate and covered with an additional layer of collodion in order to increase contrast. FIGURES 3 and 4 show examples of the findings and it may be seen that many particles have a dense inner body surrounded by a membrane. Other particles consist essentially of two concentric circles; occasionally the inner circle contains a small

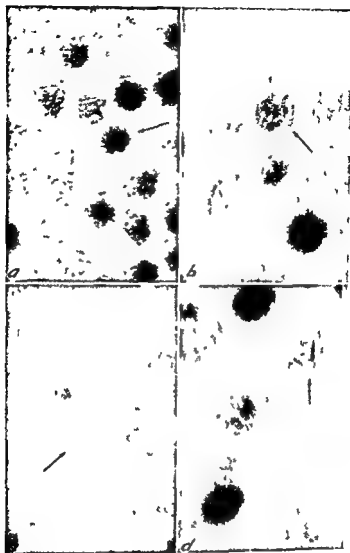


FIGURE 4
Type 3 aden
membrane
membranes
 $\times 160,000$

granule. While these different forms may have resulted from cuts at different levels in the particles, it seems more likely that different forms of the particles exist.

It was pointed out that present methods do not permit direct infectivity tests of particles in sectioned cells so that the true viral nature of intracellular particles cannot be tested. Nevertheless, it has been shown^{19, 20} that purified

pellets of avian leukosis viruses contained particles with internal structure resembling closely the virus like particles seen in sectioned cells. This method of comparing the structure of particles that can be tested for infectivity with particles visualized in sections seems to offer an approach to the problem as



Fig. 5. Electron micrograph of HeLa cell 21 hours after infection with Type 2 poliovirus. There are the granules within the infected nucleus and in localized areas outside of the nuclear membrane. (X45,000)

how to ascertain the infectivity of intracellular particles. However in recently reported sections of pellets of purified poliovirus, no membrane was found to surround the particles.² It is not clear at present whether the lack of a membrane was the result of difficulties in resolution of this very small viral particle or whether the membrane does not exist.

In recent studies of HeLa cells infected with poliovirus we have employed a Type 2 strain both before and after adaptation in HeLa cells by serial passage. Sections were prepared as in previous studies⁴ and also by the method of Watson⁵ noted above. FIGURES 5 and 6 show distorted nuclear membranes



FIGURE 6 Electron micrograph of HeLa cell 21 hours after infection with Type 2 poliovirus. Above the distorted nucleus is an area containing fine granules similar to those present inside the nucleus. X6322

and many small particles or granules within the nucleus and in large extra nuclear clusters. Examination of such particles under high magnification not only showed no internal structure but failed to show smooth margins or regular shape. This experience is similar to that of Kallman *et al.*⁷ who found no viruslike particles in tissue cultures of monkey kidney cells infected with polio-

virus. These investigators also calculated from titrations of the culture fluids that too few viral particles were present to be identified by electron microscopy. It is known moreover, that poliovirus is released rapidly from cells in tissue culture,²²⁻²⁴ so that the number of viral particles remaining in the cells might be expected to be minimal in number. Conversely, adenoviruses are not easily released from cells,²⁵ and viruslike particles are readily found in cells of in

cell system under study.

The failure to find viruslike particles in cells undergoing a severe cytopathogenic effect from infection with poliovirus is consistent with the view of Ackermann *et al*²⁶ that cytopathogenicity is at least partially a function of the toxic effect of the virus. A similar view arose from our finding that viruslike particles were found in only a minority of HeLa cells inoculated with adenovirus.

place. If a large part of the cytopathogenicity of some viruses in tissue culture is the result of toxic action as appears to be the case in these examples, it may be worthwhile to speculate concerning possible cell virus systems in which a cytopathogenic effect would not take place because the virus lacked toxicity for the particular cells employed, but might be visualized inside of cells by electron microscopy.

Finally, the problem of artifacts in the electron microscopic study of lesions in cells induced by viral infection must be considered. Moreover, variations from preparation to preparation may make it difficult to compare infected and uninfected cells. In HeLa cells the cytoplasm usually shows structures roughly the size of mitochondria; however, these do not have the typical cristae usually needed for the identification of mitochondria. Some of such structures probably consist of mitochondria that have undergone degenerative changes as the result of some premortem influence such as viral infection, or have this appearance because of changes that have taken place during preparation of the cells for electron microscopy.²⁷ In addition, we think that many of such structures are the inclusion vacuoles resulting from pinocytosis, since uninfected HeLa cells grown in media containing colloidal gold show particles of gold in globoid structures of the cytoplasm but not in typical mitochondria.²⁸ We have found also that similar ingestion of colloidal gold takes place in HeLa cells undergoing cytopathogenic effect from the action of adenovirus.

Acknowledgments

We are indebted to Frank Horsfall, Jr. for supplying the pneumonia virus of man and for confirming infection of man by serologic test. We thank Barbara Watson for her help with the details of the fluorescent antibody method and Albert Coons for a generous gift of fluorescein amine. The photographic prints were prepared by Cameron Lewis.

RELATION OF VIRUSES TO INCLUSION BODIES

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In this paper, I shall discuss first the basic reaction patterns of cells to viral infections as they are observed under the conventional light microscope, and attempt to correlate these patterns with some of the data obtained by the use of newer techniques. Second I shall review certain observations on the intracellular multiplication of rickettsiae which may be pertinent to the analogous problem of the cytoplasmic and nuclear multiplication of viruses.

History

The molluscum body was described in 1841 and the cytoplasmic inclusion in fowlpox (the Bollinger body) in 1873. These bodies together with other similar structures described later were long believed to be protozoa or fungi. Borrel¹ in 1904, showed that the Bollinger body was composed of myriads of minute coccoid elements each about 0.2μ in diameter. In 1920 Woodruff and Goodpasture² showed that isolated washed Bollinger bodies were infective.

The nuclear inclusions of herpes simplex were described by Lipschutz in 1921. George Hays and I in 1932 observed compact nuclear clusters of spotted fever rickettsiae in tissue cultures and stressed the resemblance of these intranuclear aggregates to the nuclear inclusions seen in certain viral infections.³

Thus the general concept that certain viral inclusions, both cytoplasmic and nuclear might be colonies of viral particles gradually gained momentum and in the case of several of the larger viruses was confirmed by direct observation although the occurrence of a matrix in which the elementary bodies were embedded somewhat complicated the picture.⁴ The nature of the homogeneous inclusions associated with the smaller viruses remained in the realm of speculation and has only recently been partially clarified by the use of the electron microscope.

Meanwhile new virological concepts were being formulated and developed in the general areas of the host-parasite relationship and in the method of multiplication notably the diversion of enzymes of their host cells to viral replication the multiplication of the smaller viruses by a mysterious method differing from that of binary fission the breakdown of virus particles into subunits that were reduplicated separately and later combined to form mature

the important questions obviously are what enzymes are diverted and how and in what direction they are diverted. By what type of biochemical persuasion does the virus particle after entering its host cell divert its enzymatic machinery to its own replication? At present we possess several new techniques for asking specific questions in this field of inquiry, and we are getting

answers to these questions. To be sure, each answer raises several new ques

Basic Reaction Patterns of Cells to Viral Infection

Since all cytopathic reactions of cells to viral infection are interrelated, it seems desirable to discuss them as a whole before considering the special case of inclusion body formation. Cellular alterations may be observed in undisturbed animal tissues or in cells isolated in tissue culture. The latter method has the advantage of eliminating those modifications of the reaction patterns resulting from such factors as antibody formation and nonspecific inflammation. Certain specific alterations, however, notably neoplasia and hypoplasia, can be observed only in the intact animal.

Under the light microscope, virus infected cells may appear unaltered, or may show (1) staining alterations, (2) assumption of spherical shape, (3) necrosis, (4) hypertrophy, (5) giant cell formation, (6) hypoplasia, (7) metaplasia, (8) hyperplasia, (9) neoplasia, or (10) inclusion body formation. The various alterations may be observed in a wide variety of combinations that may occur simultaneously or sequentially. The formation of syncytial giant cells and inclusion bodies, for example, may appear to be simultaneous, but staining alterations, hyperplasia (proliferation), inclusion body formation, and necrosis often occur sequentially. Sequential changes are best studied in tissue cultures, but the various developmental stages often may be observed in infected animal tissues and correlated with observations made in tissue cultures. There are some obvious advantages in studying intact cells in living animals, since cells in tissue culture are under highly artificial conditions. It is, in fact, rather surprising that there is, in general, excellent correlation between observations made under such different conditions. The principal difference is that latently infected animal tissues without inclusions or necrosis may develop inclusions and necrosis when grown in tissue culture, or when tissue cultures are inoculated with their cell free extracts.

The absence of cytological change in virus infected cells is readily explained by assuming that relatively few virus particles are uniformly distributed in

testicular tissue from one rabbit into the testes of another, a technique injury is indicated by the occurrence of nuclear inclusions and necrosis. Staining alterations alone usually represent an early stage in development.

formation is particularly prominent in measles, varicella, and canine distemper, but it should be noted that this process in the living animal is seen only in certain organs and in certain developmental stages of the infection. Hypoplasia (arrested embryonic development) is exemplified by the cerebral lesions

viral diseases. Hyperplasia may be diffuse and of moderate degree (the neuroglial cell proliferation in chronic inclusion encephalitis) or localized and extensive, as in molluscum contagiosum or infectious rabbit papillomatosis. Malignant neoplasia initiated by viral infection has been found only in lower animals.

Factors Influencing the Reaction Pattern

Duran Reynals⁷ has shown that the Rous sarcoma virus may cause necrosis in young chickens while older chickens react to the virus by developing malignant neoplasia. Similarly, the reaction pattern may be influenced by genetic constitution (either of the virus or of the host), hormones, antimetabolites and by a variety of enzymatic inhibitors and activators. (Of particular interest are α -interferon and compounds such as ribavirin, for example.) Pre- and postinfective changes with the formation of virus nucleoproteins, but in no instance has the precise mechanism been determined.

Inclusion Body Patterns in Viral Infections

The visualization of relatively minute aggregates of elementary bodies by the electron microscope probably makes it necessary to broaden our definition of the term inclusion body. Conventional inclusion bodies, however, as seen under the light microscope, range in diameter from 1 or 2 to 20 or more μ in greatest diameter. They may be found in association with any of the other cytological alterations described above including in lower animals neoplasia.

In about one half of the known viral infections of man and lower animals, inclusion bodies have not been observed. As suggested above, this is probably because there are no visible aggregates of elementary bodies or of their component parts. Inclusion bodies may be present in cells without other cytopathic changes but this probably represents early infection, necrosis or atrophy presumably follows, although the possibility of eventual recovery has not been excluded.

Inclusions may occur in the nucleus, in the cytoplasm, or in both. On the basis of the colony formation theory, one would be tempted to associate these three patterns with preferential multiplication in the nucleus or cytoplasm because of different metabolic requirements. Observations with the light microscope do not justify this hypothesis, however, since, at least in the case of small viruses, a cell showing only a nuclear inclusion might contain large numbers of uniformly distributed virus particles in its cytoplasm and the reverse situation also might result.

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The absence of cytological change in virus infected cells is readily explained by assuming that relatively few virus particles are uniformly distributed in cytoplasm or nucleoplasm. An example of this situation is found in virus III infection in the testis of the rabbit, no cellular alterations are seen unless the degree of infection is magnified by the artificial method of injecting homogenized testicular tissue from one rabbit into the testis of another, whereupon cellular injury is indicated by the occurrence of nuclear inclusions and necrosis.

Staining alterations alone usually represent an early stage in development

infected cells (not merely their inclusions) were thought to be protozoa⁵ until Goodpasture,⁶ in 1921, published evidence that these peculiar cells were derived from normal cells by a type of cytomorphosis. Giant cell

formation is particularly prominent in measles, varicella, and canine distemper, but it should be noted that this process in the living animal is seen only in certain organs and in certain developmental stages of the infection. Hypoplasia (arrested embryonic development) is exemplified by the cerebral lesions resulting from intrauterine infection of infants with the salivary gland virus. Squamous metaplasia is prominent in the bladder of minks or ferrets suffering from distemper and in pulmonary alveolar duct epithelium in a number of viral diseases. Hyperplasia may be diffuse and of moderate degree (the neuroglial cell proliferation in chronic inclusion encephalitis) or localized and extensive as in molluscum contagiosum or infectious rabbit papillomatosis. Malignant neoplasia initiated by viral infection has been found only in lower animals.

Factors Influencing the Reaction Pattern

Duran Reynals⁷ has shown that the Rous sarcoma virus may cause necrosis in young chickens while older chickens react to the virus by developing malignant neoplasia. Similarly the reaction pattern may be influenced by genetic

of virus nucleoproteins but in no instance has the precise mechanism been determined.

Inclusion Body Patterns in Viral Infections

The visualization of relatively minute aggregates of elementary bodies by the electron microscope probably makes it necessary to broaden our definition of the term "inclusion body" to include any such aggregate, whether it is seen under greater magnification or not.

Inclusions may occur in the nucleus, in the cytoplasm, or in both. On the basis of the colony formation theory, one would be tempted to associate these three patterns with preferential multiplication in the nucleus or cytoplasm because of different metabolic requirements. Observations with the light microscope do not justify this hypothesis however since, at least in the case of small viruses, a cell showing only a nuclear inclusion might contain large numbers of uniformly distributed virus particles in its cytoplasm and the reverse situation also might result.

Certain specific cytological changes are associated with inclusion body formation. In the case of cytoplasmic inclusions, the peripheral clear zone or halo is most prominent. With Type A nuclear inclusion formation, the nuclear chromatin may be margined, often with two distinct marginal polar bodies, chromatin may disappear from the body of the nucleus, so that it appears empty except for the inclusion. Wrinkling of the nuclear membrane also is common.

In certain viral infections associated with both nuclear and cytoplasmic inclusions, the cytoplasmic bodies are stained brightly by the periodic acid Schiff (PAS) method, while the nuclear inclusions remain unstained, cytoplasmic inclusions usually remain unstained by silver methods, while nuclear inclusions are deeply stained. These reactions perhaps may be correlated with the cytoplasmic multiplication of the polysaccharide protein unit of the virus particle and with the replication of the nucleic acid protein portion in the nucleus, which has been demonstrated in certain viral infections. In salivary gland virus infected cells, localized areas of fine dustlike PAS positive material are often noted in the cytoplasm.

Electron microscopic studies of viral inclusions have in general, confirmed the colony formation theory, in that most inclusions that have been studied appear to contain elementary bodies at some stage in their development. Homogeneous bodies may appear first (perhaps representing the accumulation of macromolecular building blocks), and the virus particles, or their subunits may separate later from this matrix. In other instances, the elementary bodies multiply first, and the mature inclusion seems to be a product of their degeneration. Further studies are needed, however, since sequences are difficult to establish in killed tissue. Cinematographic studies of living cells under the electron microscope would be ideal, but this method of approach is not yet available.

Rickettsial Multiplication in Cytoplasm and Nucleus

Rickettsiae differ from the smaller and more typical viruses in their bacilluslike morphology, their multiplication by binary fission, and their sensitivity to certain antibiotics. The large viruses of the lymphogranuloma psittacosis group, however, resemble the rickettsiae in the above respects. Rickettsiae, like viruses, are obligate intracellular parasites, and separation of the two groups must be made on rather arbitrary grounds.¹⁰

Cells infected with rickettsiae show patterns similar to those seen in virus infected cells: diffuse cytoplasmic growth (rickettsiae of the typhus group), cytoplasmic clusters (*Rickettsia burnetii*), and intranuclear aggregates (rickettsiae of the spotted fever group). The latter show their intranuclear growth best in tissue cultures and in the tissues of their arthropod hosts (ticks). In addition to their massive intranuclear growth in tissue cultures, spotted fever rickettsiae also grow sparsely in the cytoplasm. Typhus rickettsiae are never seen in nuclei and neither typhus nor spotted fever rickettsiae are ever found in nucleoli. The latter structures remain clear even when nuclei are packed and even distended with spotted fever rickettsiae.

The antibiotic response patterns of typhus and spotted fever rickettsiae are identical. In guinea pigs, sulfa drugs increase rickettsial growth, in the case of spotted fever rickettsiae, the increased growth occurs particularly within

nuclei. In fertile eggs penicillin, chloramphenicol, streptomycin and the tetracycline drugs all cause marked inhibition and almost complete cessation of the multiplication of both types of rickettsiae. Para aminobenzoic acid and high incubation temperature (40°C) are rickettsiostatic for both types of rickettsiae. Cells massively infected with typhus rickettsiae are distended to two or three times their normal diameter and assume a spherical shape. Nuclei heavily infected with spotted fever rickettsiae usually retain their normal shape but frequently their diameters are doubled and there is definite margination of the nuclear chromatin.

We have found only one enzymatic modifier that shows antithetical effects on rickettsiae of the two groups. Radiation which in the fertile egg markedly enhances the growth of *Rickettsia mooseri*¹¹ (a member of the typhus group) inhibits and under certain conditions completely suppresses the multiplication of *Rickettsia akari*¹² (a member of the spotted fever group). The rickettsiostatic action on *R. akari* is noted when single doses of γ radiation are given but is most striking and complete when irradiated water, a source of continuous beta emission is injected into the yolk sac. Both the increased growth of *R. mooseri* and the inhibition of *R. akari* are roughly proportional to the dosages of radiation used.

It seems probable that these antithetical reactions to radiation may reflect a metabolic difference between the two organisms which is correlated with their different cytological growth patterns. The important effects of radiation on nucleic acid metabolism would afford a logical explanation in general terms of this correlation. Radiation is known to exert a number of different effects on cellular metabolism including the inhibition of nucleic acid synthesis, conversion of ribonucleic acid (RNA) to deoxyribonucleic acid (DNA), inhibition of oxidative phosphorylation, depolymerization of nucleic acids and inhibition of sulphydryl enzymes in general. It could be expected therefore that any particular biological effect observed would depend on the resultant of these various activities. In the case of enhancement or stimulation of rickettsial growth in fertile eggs however the almost quantitative effects of various dosages over a wide range compatible with continued embryonic development encourage us to believe that some one specific radiation effect may play the important and dominant role. By using agents that exert separately the various effects of radiation it may be possible to pinpoint this specific effect.

In studying the effects of γ radiation on the multiplication of *R. mooseri* in fertile eggs two incidental observations were made. First the rickettsiostatic effect of high incubation temperature (40°C) was reversed by radiation in irradiated eggs rickettsiae grew just as freely at 40°C as they did at 37°C without radiation.¹³ Second the rickettsiostatic action of streptomycin was reversed by γ radiation and this reversal was quantitative in nature with increasing doses of streptomycin larger doses of radiation were required to inhibit the rickettsiostatic action.¹⁴ Similar studies on *Rickettsia akari* have not yet been carried out.

Effects of Radiation on Viral Multiplication

We have studied the effect of radiation on only one virus the H₁N₂ strain of influenza A virus.¹⁵ Groups of embryonate eggs were given 250, 500, 750 and

1000 r of X radiation. Eighteen hours later, these 4 groups, together with a control group, were injected intraallantoically with the virus. Radiation at all dosage levels caused significant changes in the infectivity titer curves during the next 90 hours. The most striking effect noted, however, was the slow development and very low level of the infectivity titer that followed the administration of 750 r per egg; other dosage levels resulted in less obvious effects. It seems probable that in these experiments two or more antagonistic effects of radiation may be involved, although it also is possible that radiation may have exerted different effects on the reduplication of the viral subunits.

It would seem desirable to study the effects of radiation on a number of other viruses, selected on the basis of their multiplication in the cytoplasm, in the nucleus, or in both parts of the cell. Insofar as I am aware, studies of this type have not been carried out in a systematic manner.

Relation of Nucleic Acid Composition to Viral and Rickettsial Multiplication

Analysis of typhus rickettsiae indicates that they contain both DNA and RNA, the DNA predominating. Unfortunately, I have been unable to find reports on the nucleic acid makeup of rickettsiae of the spotted fever group. This information might furnish an important clue to the preferential localization of the two types of rickettsiae in the cytoplasm or nucleus, respectively, and also to their antithetical response to radiation.

The nucleic acid composition of a number of purified viruses has been determined. Since the outstanding difference between nucleoplasm and cytoplasm is the preponderance of DNA in the former and the almost exclusive presence of RNA (under normal conditions) in the latter, it seems possible that the preferential multiplication of virus particles (or of rickettsiae) in one or the other part of the cell may be related to this basic difference. If it were true that RNA viruses always multiplied in the cytoplasm and DNA viruses in the nucleus, the problem would be greatly simplified. Since the simple relationship obviously does not hold, some other type of relationship must be sought.

The development of DNA viruses in the cytoplasm where little or no DNA is normally present makes it clear that such viruses are able to cause profound alterations in the nucleic acid metabolism of their host cells. In the case of the bacteriophage the evidence is against the direct transfer of bacterial nucleic acid to the viral particles, although a certain proportion of bacterial DNA is available after partial breakdown for the formation of viral DNA.¹⁸ Presumably, therefore, most of the viral DNA must be constructed from smaller building blocks available in the surrounding medium. The rapidity with which some DNA viruses multiply in the cytoplasm makes one wonder what happens to the RNA normally present there. Possibly it is broken down to nucleotides, or smaller units, which are then used in building DNA according to the specifications of the newly arrived virus particle. Thus some viruses, either by virtue of an intrinsic enzymatic property, by diversion of enzymes in their host cells, or by evoking or unmasking latent host cell enzymes, may be considered to act as nucleic acid converters (RNA to DNA) when they replicate in host cell cytoplasm.

In the study of such phenomena, one is apt to look on the living molecules of the viral components as the keys to the situation. A consideration of cells

lar machinery as are their smaller and more glamorous relatives. Possibly, rickettsial multiplication may be explained on some relatively simple basis such as the segregation of nucleic acid by its incorporation in their bodies with consequent disturbance of some normal equilibrating mechanism and the initiation of a chain reaction. In the present state of our knowledge one scarcely needs to apologize for the introduction of highly speculative suggestions. In any case, the intracellular multiplication of rickettsiae may involve in a simplified form many of the problems concerned in viral multiplication and it therefore seems worthy of further study by the newer methods of analysis.

Conclusion

The nature of viral inclusions is part of a more general problem—the kinetics and biochemistry of those mysterious processes by which viruses and rickettsiae multiply within susceptible cells. This problem in turn is related to the cytology and physiology of cells under normal and pathological conditions. Even though we have at present only incomplete and often apparently conflicting fragments of information in these areas the use of new techniques of study may be expected to result in the near future in a greater degree of order and harmony. The almost incredible progress made in recent years certainly justifies this assumption and there is every reason to believe that this progress will continue at an accelerated rate not only by the accumulation of more data but by the correlation and synthesis of data already in our possession.

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